Discogenic Back Pain

The Induction and Prevention of a Pro-inflammatory Cascade in Intervertebral Disc Cells *in vitro*

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SUMMARY

Low back pain (LBP) is a prevalent symptom that more than 80% of the population experience once in their lifetime. This can lead to severe impairment of the workaday life and cause enormous costs in the society. Because LBP mostly appears as a non-specific back pain symptom, provoked by the spine or its environment, the evaluation of the source is bearing some challenge. Whereas the pathomorphological source of pain is well defined in the specific spinal pathology, such as in the case of a scoliosis or sciatica, finding a correlation between the source of pain and a certain abnormality is difficult in non-specific LPB symptoms. This is accompanied by the disadvantage of finding a suitable treatment. One possible source of LPB represents the intervertebral disc (IVD), which can alter from a pain free (asymptomatic) to a painful (symptomatic) IVD during degeneration, leading to so called discogenic back pain. Provocative discography is to date the only means to assign LBP to a degenerated disc, with its usage being under dispute.

The IVD has an important function as a shock absorber, as there is a high load on the spine. During a lifetime, our IVD becomes degenerated which means its matrix is more catabolized then anabolized, leading to an overall matrix breakdown and decreased quality of the IVD. The matrix consists of long protein chains and sugars, responsible for the ability to attract water, comparable to a sponge. Due to the reduction and loss of these main components during degeneration, the IVD loses height and we get smaller during aging. This is a normal process which is pain free in most cases, meaning asymptomatic. But there is a certain subpopulation complaining about pain without showing any special pathomorphological changes. Thus far it was demonstrated that symptomatic degenerated IVDs produce more cytokines (IL-6, IL-8, IL-1β, TNF-α) and that these molecules are able to provoke pain sensation directly. The first part of this thesis aims to identify factors leading to a pro-inflammatory cascade in a degenerated IVD as well as the involved pathways.

The matrix of the IVD consists of abundant structure proteins such as collagen or fibronectin as well as of a special sugar, the hyaluronic acid. During disc degeneration,

these huge molecules become fragmented and catabolized. In this study we were able to show that hyaluronic acid fragments (fHA) provoked a pro-inflammatory and catabolic cascade in IVD cells *in vitro*. With gene silencing and inhibition of activity, we could detect TLR2 to be engaged in the up-regulation of IL-6 synthesis in fHA treated IVD cells. Furthermore, inhibition experiments demonstrated an involvement of the MAP kinases ERK and JNK, two classical enzymes in inflammatory reactions. Surprisingly, we could not observe any NF-κB involvement, another important pathway in inflammatory signalling.

The second part of this work is dedicated to potential anti-inflammatory and anti-catabolic herbal agents for a possible application in the treatment of discogenic back pain. Resveratrol, curcuma (curcumin) as well as triptolide showed anti-inflammatory and anti-catabolic effects on IL-1β prestimulated IVD cells *in vitro*. Interestingly, all of these substances had no influence on the NF-κB pathway. Curcumin, an ingredient of curcuma, reduced JNK phosphorylation and thereby its activation. Triptolide instead limited p38 and ERK phosphorylation. Resveratrol did not show any effect on the investigated pathways (NF-κB and MAP kinases), but exhibited an analgetic effect *in vivo,* conducted in a rat model.

All tested substances showed promising effects for a possible use for the treatment of symptomatic degenerated discs, either by an intradiscal or epidural injection, e.g. by usage of an appropriate slow release system.

ZUSAMMENFASSUNG

Rückenschmerzen gehören zu den am häufigsten auftretenden Symptomen in unserer Gesellschaft. Weltweit leiden über 80% mindestens einmal in ihrem Leben an Rückenschmerzen. Diese Schmerzen können zu einer grossen Beeinträchtigung im alltäglichen sozialen und beruflichen Leben führen und verursachen dabei auch sehr hohe Arztkosten. Es gibt mehrere Ursachen die zu Rückenschmerzen führen können, und es ist deshalb nicht immer einfach, die eigentliche Quelle des Schmerzes zu finden. Selten ist die direkte Verbindung zwischen Schmerzäusserung und spezifischem Krankheitsbild so offensichtlich wie im Falle von definierten Wirbelsäulenerkrankungen, z.B. Verkrümmung der Wirbelsäule (Skoliose) oder Verengung des Wirbelkanals (Ischiassyndrom). Unspezifische Erkrankungen der Wirbelsäule und deren Umgebung die zu Schmerzempfindungen führen können treten im Allgemeinen häufiger auf und erschweren die Suche nach einer passenden Behandlung. Eine mögliche Ursache des Schmerzes kann eine degenerierte Bandscheibe (englisch: intervertebral disc = IVD) sein, die sich von einer schmerzfreien (asymptomatischen) zu einer schmerzhaften (symptomatischen) Bandscheibe verändern kann (discogenic back pain). Die provokative Diskographie ist zur Zeit die einzige – jedoch umstrittene - Methode, bandscheibenbedingte Rückenschmerzen als solche zu identifizieren.

Unsere Bandscheibe hat eine wichtige Stossdämpferfunktion, denn es wirken enorme Kräfte auf unsere Wirbelsäule. Im Verlaufe eines Lebens degeneriert die Bandscheibe, d.h. ihre Matrix wird mehr abgebaut als aufgebaut, wodurch sich die Qualität der Bandscheibe verringert. Diese Matrix besteht aus vielen langkettigen Eiweissen und Zuckern, die viel Wasser anziehen können, vergleichbar mit einem Schwamm. Durch den Abbau und Verlust dieser Hauptbestandteile kommt es bei einer Degeneration zu einer immer flacher werdenden Bandscheibe und wir werden mit dem Alter immer kleiner. Dieser Prozess ist in der Regel schmerzfrei, also asymptomatisch. Nun gibt es aber Patienten, die über Schmerzen im Rücken klagen, jedoch keine besondere pathomorphologische Merkmale aufweisen. Bisher konnte gezeigt werden, dass symptomatisch degenerierte Bandscheiben einen erhöhten Zytokinlevel aufweisen (IL-6,

IL-8, IL-1β, TNF-α) und dass einige dieser Botenstoffe direkt Schmerzempfindungen auslösen können. Welche besonderen Faktoren in einer degenerierten Bandscheibe zu dieser proinflammatorischen Stimulierung führen und welche Signalwege dabei involviert sind, ist der erste Teil dieser vorliegenden Arbeit gewidmet.

Die Matrix der Bandscheibe besteht neben Strukturproteinen wie Kollagen oder Fibronektin auch aus einem besonderen Zucker, der Hyaluronsäure. Während der Degeneration der Bandscheibe werden diese langkettigen Moleküle in kleinere Einheiten fragmentiert und abgebaut. Wir konnten *in vitro* zeigen, dass Hyaluronsäurefragmente (fHA) eine proinflammatorische wie auch katabolische Wirkung auf Bandscheibenzellen aufweisen. Dabei wurde TLR2 durch Inaktivierung auf Genebene wie auch durch Blockierung seiner Aktivität auf Proteinebene als ein Rezeptor identifiziert, der bei der Behandlung der Zellen mit fHA an der Ausschüttung von IL-6 beteiligt zu sein scheint. Mittels Inhibitionsexperimenten konnten wir weiter zeigen, dass dieses Signal hauptsächlich über die MAP Kinasen ERK und JNK vermittelt wird, zwei klassische Enzyme, welche die Zellen bei Entzündungsreaktionen aktivieren. Erstaunlicherweise konnten wir keine Beteiligung von NF-κB detektieren, ein weiterer wichtiger Signalweg in Entzündungsreaktionen.

Der zweite Teil der Arbeit widmete sich pflanzlichen Wirkstoffen. Diese wurden auf ihre antiinflammatorische wie auch antikatabolische Wirkung hinsichtlich eines möglichen Einsatz in der Behandlung gegen schmerzhafte degenerierte Bandscheiben getestet. Resveratrol, Curcuma sowie auch Triptolide, alles Substanzen pflanzlicher Herkunft, zeigten *in vitro* antiinflammatorische wie auch antikatabolische Wirkung auf mit IL-β vorstimulierte Bandscheibenzellen. Die Substanzen zeigten interessanterweise keinen Einfluss auf den NF-κB Signalweg. Curcumin, einer der Inhaltsstoffe von Curcuma, reduzierte jedoch die JNK Phosphorylierung und somit die Aktivierung dieses Signalwegs. Triptolide hingegen hatte einen hemmenden Einfluss auf die Phosphorylierung von p38 und ERK. Resveratrol zeigte keine Wirkung auf die untersuchten Signalwege (NF-κB und MAP Kinasen), jedoch konnte *in vivo* ein analgetischer Effekt nachgewiesen werden, durchgeführt in einer Studie an Ratten.

Für einen möglichen Einsatz in der Behandlung von symptomatisch degenerierten Bandscheiben zeigten sich alle drei Substanzen als interessante Kandidaten, die entweder intradiscal oder epidural (möglicherweise in einer slow-release Formulierung) eingesetzt werden könnten.

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ABBREVIATION

AP-1 = activating protein-1

AD = ADAMTS = a disintegrin and metalloproteinase with thrombospondin motifs

BSA = bovine serum albumin

DRG = dorsal root ganglion

DTT = 1,4-Dithio-DL-threitol

DD = disc degeneration

DDD = disc degenerated diseases

ECL = enhanced chemiluminescence reaction

ECM = extracellular matrix

EDTA = ethylendiamintetra-acetat

ELISA = enzyme linked immunosorbent assay

ERK = extracellular signal regulated kinase

FACS = fluorescence activated cell sorting

fHA = fragmented hyaluronic acid

GAG = glycosaminoglycan

GPCR = G protein-coupled receptor

HA = hyaluronic acid

HAS = hyaluronic acid synthase

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMWHA = high molecular weight hyaluronic acid

HRP = horseradish peroxidase

Hyal = hyaluronidase

IHABP = intracellular hyaluronic acid binding protein

IVD = intervertebral disc

JNK = c-Jun aminoterminal kinase

LBP = low back pain

MAP kinases = mitogen-activated protein kinases

MDR = multi-drug resistance

MMP = matrix metalloproteinase

MSC = mesenchymal stem cells

NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells

OA = osteoarthritis

PBMC = peripheral blood mononuclear cells

PBS = phosphate-buffered saline

PMSF = phenylmethanesulfonyl fluoride

PVDF = polyvinylidene fluoride

RA = rheumatoid arthritis

RHAMM = receptor for hayluronan mediated motility

RT = room temperature

RTK = receptor tyrosine kinase

SDS-PAGE = sodiumdodecylsulfat-polyacrylamid-gelelectrophorese

TBS = tris-buffered saline

TIMP = tissue inhibitor of metalloproteinase

TLRs = toll-like receptors

VEGF = vascular endothelial growth factor

WCE = whole cell extract

INTRODUCTION

1. Low back pain

1.1. Symptoms

Back pain is a prevalent symptom that more than 80% of the population experience once in their lifetime. The most common symptoms are neck and low back pain (LBP), which can lead to a severe impairment of the workaday life and cause enormous costs in the society. Because LBP mostly appears as a non-specific back pain symptom, provoked by the spine or its environment, determining the source of this problem possess a serious challenge. Whereas the pathomorphological source of pain is well defined in the specific spinal pathology, such as in the case of a scoliosis or sciatica, finding a correlation between the source of pain and a certain abnormality is often difficult in non-specific LPB symptoms. This is accompanied by the disadvantage of finding a suitable treatment. The development of LBP is a multi-factorial process in which all spinal structures as well as aspects such as age-related changes and life style can contribute. Physiological and psychological aspects have to be considered as well when we are questioning the origin of the pain sensation. The spine as a source of pain involves the vertebrae, the facet joints, the muscle, the ligaments or the intervertebral disc (IVD), while age related changes of the spine can appear as a loss of muscle mass, decreased structural strength of the vertebral body, cartilage endplate calcification and ossification, facet joints osteoarthritis or a decreased tensile strength of ligaments. Another possible source of LPB represent the intervertebral disc (IVD), which can alter from a pain free (asymptomatic) to a painful (symptomatic) IVD during degeneration, which is then called discogenic back pain.

Individual risk factors to generate LPB can be age, gender, body weight, general health status, heredity, smoking or a sedentary lifestyle as well as morphological factors such as e.g. scheuermann disease, disc herniation or, as already mentioned, a degenerated IVD. Of course, all these risk factors do not necessarily lead to pain sensation [1].

The diagnostic triage further classifies LBP either to specific spinal pathology (specific structural pathology is consistent with the clinical picture), nerve root pain/radicular pain or non-specific low back pain. Pain can additionally differentiated into nociceptive pain, neurophathic pain, functional pain or inflammatory pain [2]. Nociceptive pain is provoked by a noxious peripheral stimuli like heat, cold, mechanical force or chemical irritants to the nociceptors whereas direct damage or disease of neurons in the peripheral or central nervous system is typical for neurophatic pain. The functional pain is an abnormal responsiveness or function of the nervous system, and there are no neurological or peripheral abnormalities detectable. The inflammatory pain arises from injured tissue and inflammatory mediators such as cytokines, provoking pain sensation at nociceptors [1, 3]. For a diagnostic evaluation, it is important to consider all the above mentioned possible sources of LBP in order to find an appropriate treatment for the patient.

1.2. Treatments

Treatment options for LPB are pharmacological, physiotherapy and operative applications. Opioids, non-opioid, non-steroidal anti-inflammatory drugs and adjuvants are applied as pharmacological drugs. Among the most commonly used opioids are morphine, hydromorphone, methadone, oxycodone, oxymorphone and fentanyl, which are mostly applied in cases of acute severe pain. The most frequently used non-opioid analgesic is paracetamol. The non-steroidal anti-inflammatory drugs are e.g. celecoxib, rofecoxib, valdecoxib, used for inflammatory pain treatment. They mainly block the synthesis of prostaglandine E_2 (PGE_2), responsible for the direct pain sensation, but have the disadvantage of also inhibiting the activities other prostaglandins, which have tissue protective functions. Often, antidepressants, anticonvulsants, anxiolytics, muscle relaxants and sleep-promoting medication are also administered as adjuvants.

For invasive intervention for LBP, spinal injection of selective nerve root blocks such as bupivacaine in combination with methylprednisolone or lidocaine with betamethasone are used as a diagnosis to differentiate symptomatic and asymptomatic alteration and as a treatment of various spinal disorders as an adjunct to non-operative care. Corticosteroids are used for epidural injections with the aim to diminish the inflammatory component of a neural compromise. Provocative discography is used to distinguish asymptomatic from

symptomatic disc degeneration as an alteration is not detectable with classical imaging technique like MRI. It is applied in patients who are potential candidates for surgery. Thereby, a non-ionic contrast agent is injected intradiscally into the suspected diseased pain evoking IVD. During leakage, the patient has to describe the pain sensation, which is thought to arise through the expulsion of pathological metabolites such as neuropeptides or cytokines from the disc to the outer part of the annulus, resulting in irritation of the nerve endings. A healthy IVD that does not provoke pain serves as an internal control. Risk factors of this treatment are infections, allergic reactions or nerve root sheath injuries. This diagnostic value also remains a matter of debate as long term studies have shown only moderate pain relief. Furthermore, a study by Carragee *et al.* [2009] could demonstrate that the applied discography technique, puncturing the intervertebral disc using a small gauge needle, led to a greater progression of disc degeneration compared to control (non-punctured) [4]. Thereby the healthy disc which serves as a control would be affected. Beside the loss of disc height, this method also seems to accelerate disc herniation.

Surgical treatments for degenerative lumbar diseases, spinal fusion or disc replacements are performed if a non-operative method is not helpful. Thereby, either autologous bone from anterior or posterior iliac crest (as cancellous bone, corticocancellous bone chips or tricortical bone blocks), allograft bone or bone graft substitutes (such as calcium phosphates and demineralized bone matrix) are considered for the disc replacement. However, in the long term, these treatments have not been proven to be more effective than alternative treatment in cases of severe chronic LBP. In addition, physical therapy for LBP, and staying as active as possible, can improve the pain relief and function melioration in the short term, but also lacking evidence for improvement for long term [1].

To better understand the sources and mechanism leading to LPB, it is important and worthwhile to proceed the investigations on LBP and thereby revealing new and appropriate treatments.

2. Intervertebral disc

2.1. Structure

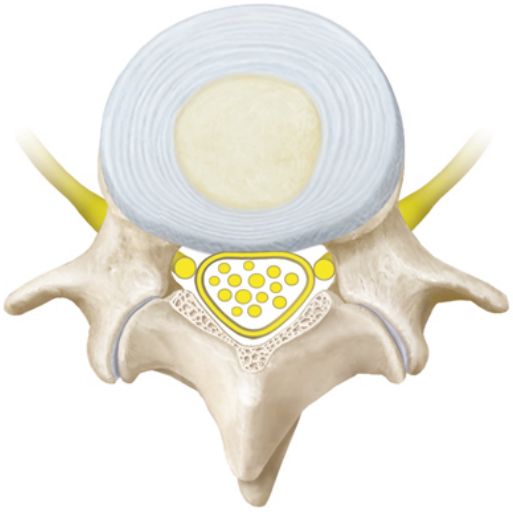


Figure 1. Schematic axial illustration of the IVD showing the NP (inner part) and the AF (outer part) (From Norbert Boos, Max Aebi: Spinal Disorders; Fundamentals of Diagnosis and Treatment).

The human spine consists of a total of 23 IVDs, which lie between the vertebral body, bordered by two end plates (hyaline cartilage), forming the integral part. The main structure of the IVD is formed by the nucleus pulposus (NP) and the annulus fibrosus (AF) (Figure 1) and is an important tissue for dissipating the high loads of the spine. Nutritional substances reach the disc by passive diffusion through the end plates [5, 6] as it is an avascular and aneural tissue, except at a sparse penetration of capillaries and nerves into the outer part of the AF [7, 8]. The IVD cells are embedded in an acidic pH and have to deal with a low oxygen supply (2%). It is reported that IVD cells can even remain viable for many days without oxygen [9, 10]. Cells form only 1% of the disc volume and are environed by a dynamic matrix structure. This matrix experiences an overall degradation during aging, resulting in a weakened ability to retain water, an important feature in the functionality of the IVD as a shock absorber. Besides water as a major component of the IVD, the extracellular matrix (ECM) (Figure 2) consists of abundant proteoglycans like aggrecan, lumican, biglycan, decorin and fibromodulin, important key players for the tissue hydration, keeping thereby the pressure within the

disc to dissipate the high loads on the spine. Important structure proteins are collagen (predominantly collagen I and II) and elastin, responsible for stability and tension of the spine. Glycosaminoglycans (GAGs) such as hyaluronic acid, chondroitin sulfate and keratan sulfate are mainly responsible for the retention of the water within the IVD due to their highly negative charge. Main glycoproteins in the IVD are fibronectin and amyloid, one function may be (beside others) to regulate fibril thickness.

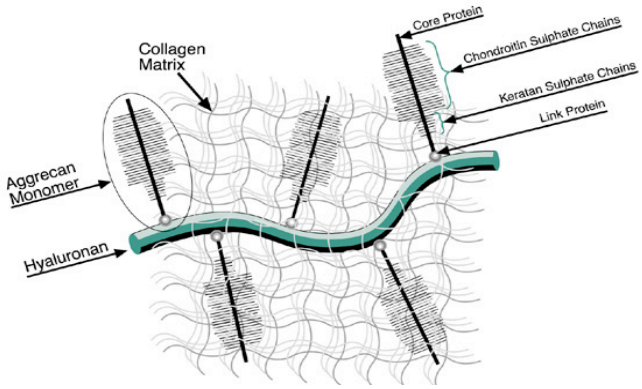


Figure 2. Simple schematic representation of some important components of the IVD ECM.

The AF (fibroblast-like cells) defines the outer part of the IVD. It consists of about 60% collagen which are organized as fibres (major type I collagen) to ensure stability in the spine. These fiber bundles are arranged in a criss-cross pattern [5]. The NP (chondrocyte-like cells) is a gelatinous fluid consisting of approximately 30% of randomly organized collagen fibres (more type II collagen), possessing more proteoglycans than the AF. The NP provides flexibility and functions as the shock absorber of the spine. For the development and differentiation to AF and NP, notochordal cells seem to play a leading role [11]. Some studies provide evidence that NP cells are notochordal cells in origin [12, 13].

The determination of typical markers for NP and AF cells for the discrimination has not been very successful so far. One study for instance tried to overcome this problem by comparing collagen I (Col-I), collagen-II (Col-II) and aggrecan synthesis ratio of NP and AF cells with articular chondrocytes (AC). They could only show a different distribution of these structure proteins [14]. Minogue *et al.* [2010] [15] as well as Power *et al.* [2011]

[16] tried to identify genes that could be used to distinguish NP cells from AC and AF in bovine and human respectively, where they came up with several genes either to be up or down regulated. For a fast and easy characterization and discrimination of NP and AF cells, a specific marker is unfortunately still missing.

As the ECM of the IVD is a very dynamic structure, it gets constantly anabolized and catabolized. Key players in this action are degrading enzymes such as matrix metalloproteinases (MMPs), aggrecanases, cathepsin and hyaluronidases (Hyals) [5, 17].

2.2. Degenerated intervertebral disc

Figure 3. Representation of the degeneration of the IVD. Left side shows a healthy young disc, right side demonstrates an aged degenerated disc. (Pictures provided by Norbert Boos).

During aging, the IVD is degenerated and thereby suffers a significant loss in disc height (Figure 3). This process of disc degeneration already begins early on in life (20 yrs of age) and is characterized by a higher catabolism then anabolism of the disc tissue leading to an overall matrix breakdown. The degeneration is also accompanied by a calcification of the endplates [18], which might contribute to the reduced nutrient transport, measured to be present in degenerated IVDs in a study of Grunhagen *et al.* [2006] [6]. Not only is aging a factor leading to the degeneration of the disc tissue. As there is a high mechanical demand on the IVD, also mechanobiological effects have to be considered, as well as the change of osmotic pressure as its consequence. Compression on the disc leads to shearing and tensile stresses and radial expansion. As resident cells interact with matrix compounds, the loads which are distributed on the proteoglycan-collagen rich ECM will

lead to a cellular response. This may contribute to tissue failure, depending on loading type, magnitude or duration [19]. Whereas low frequency of compression and high stress on a motion segment in a rodent model led to an increase in proteoglycan content [20], high frequency of loading increased mRNA levels of catabolic enzymes like MMPs or aggrecanases. Several studies in human have also confirmed a general up-regulation of mRNA and protein synthesis of degrading enzymes such as MMPs [21-23] or aggrecanases [24-27] during disc degeneration. In addition, results from a study using a rabbit model of intervertebral disc degeneration demonstrated that the expression of endogenous inhibitor of MMPs, named tissue inhibitor of metalloproteinase 1 (TIMP-1), remained low during degeneration process [28]. The expected consequence of these findings fits with the observation of different other studies documenting an accumulation of matrix degradation products such as fragmented fibronectin [29, 30] and aggrecan [27, 31, 32] in degenerated discs. Furthermore, decreases in aggrecan and collagen II mRNA level have been observed in a rabbit model of IVD degeneration. This loss of aggrecan may support further degeneration of the IVD, as aggrecan has been shown to protect cartilage collagen from proteolytic cleavage [33]. Further studies by Antoniou and Hollander *et al.* [1996] showed denatured collagen type II and a loss of proteoglycans are more prevalent in a degenerated disc [34-36] resulting in a loss of hydration and osmotic pressure [37]. Degenerated IVDs have also been reported to be greater innervated [38, 39], but whether it is more vascularized or not is a current debate as different studies show opposite results [39, 40]. The activated innervations could be partly due to the loss of aggrecan during degeneration as an *in vitro* study showed that human IVD aggrecan inhibited nerve growth [41]. Furthermore, an increased NO production was observed [42]. It is known that NO is able to react together with superoxide to form peroxynitrite, which in turn has been shown to induce MMP expression [43]. Degenerated and aged IVDs also show a higher distribution of TNF-α, its receptor and the activating TNF-α-converting enzyme [44] as well as higher level of IL-1β and IL-1β receptor [45]. In addition, a higher formation of oxidation products have been detected. Specifically, levels of advanced glycation endproducts (AGE) [46] such as carboxymethyl-lysine (CML) together with a high AGE receptor (RAGE) expression level [47] were shown to be increased compared to a young disc. Furthermore the loss of notochordal cells seems to

be implicated in the degenerative process of the IVD as demonstrated by Erwin *et al.* [2011]. They could show that these cells protect NP cells from apoptosis and that they lead to an up-regulation of genes contributing to anabolic activity and matrix protection [48].

Several studies exist which investigated the risk factors for disc degeneration. A more frequent disc degeneration was observed in smokers [41] and individuals with reduced physical activity [49]. In general we can say that besides a genetic influence, also environmental factors have an influence on disc degeneration, as discussed by Battié [1995] [50]. This is further supported by a longitudinal MRI imaging investigation of lumbar disc degeneration in asymptomatic individuals conducted by Elfering *et al.* [2002] to investigate risk factors for the development or deterioration of lumbar disc degeneration [51]. They could show in a 5-year follow up assessment that the extent of disc herniation, lack of sports activities and night shift work were significant predictors for disc degeneration.

2.3. Symptomatic degenerated intervertebral disc

There is a certain patient subpopulation developing pain sensation during degeneration of the IVD, leading to so-called discogenic back pain. As the morphology of these symptomatic discs is the same as within the asymptomatic IVDs and no alteration is detectable with classical imaging technique like MRI, provocative discography is often used as a method to identify the source of pain. In fact, it is currently the only means to differentiate symptomatic from asymptomatic disc degeneration. However, the accuracy of this test is still challenged, as clinical outcomes could not prove a significant melioration of pain relief after removal of the suspected diseased disc [52, 53].

What contributes to a symptomatic degenerated disc is still elusive. So far it has been shown that symptomatic IVDs secrete high levels of PGE_2 as well as pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 [44, 54-56], demonstrated e.g. by the increased TNF-α positive cells measured in patients with degenerative disc diseases and LBP [57]. A study by Lee *et al.* [2009] could detect a significant higher level of TNF-α and IL-8 in patients with degenerated disc diseases compared to the group with herniated

nucleus pulposus [58]. Current findings of elevated cytokines in disc degenerated diseases (DDD) are shown in table 1.

Table 1: Elevated expression level of cytokines in disc degenerated diseases (DDD).

Elevated cytokine in DDD	Detection of	Comparison to	Reference
IL-1β	Protein	control	Akyol *et al.* [2010] [56]
	mRNA + protein	degree of DD	Le Maitre *et al.* [2007] [45]
TNF-α	Protein	HNP	Lee *et al.* [2009] [58]
	Protein	Age + degree of DD	Weiler *et al.* [2005] [59]
	Protein	control	Akyol *et al.* [2010] [56]
	Protein	degree of DD	Dongfeng *et al.* [2011] [57]
	Protein	Age + degree of DD	Bachmeier *et al.* [2007] [44]
	mRNA + protein	degree of DD	Le Maitre *et al.* [2007] [45]
IL-2	Protein	control	Akyol *et al.* [2010] [56]
IL-4	Protein	control	Akyol *et al.* [2010] [56]
IL-6	Protein	Sciatica	Burke *et al.* [2002] [60]
IL-8	Protein	Sciatica	Burke *et al.* [2005] [60]
	Protein	HNP	Lee *et al.* [2010] [58]
IL-10	Protein	control	Akyol *et al.* [2010] [56]
IL-12	Protein	control	Akyol *et al.* [2010] [56]
IL-17	Protein	control	Shamji *et al.* [2010] [61]
IFN-γ	Protein	degree of DD + scoliosis	Cuellar *et al.* [2010] [62]

control=age matched autopsy, DD=disc degeneration, HNP=herniated nucleus pulposus

These cytokines are widely discussed to have the potential to provoke pain sensation [63-67]. For instance, disc herniation commonly results in radicular pain, which is considered as a result of the mechanical compression and chemical irritation of the dorsal root

ganglion (DRG) and/or spinal nerve root. Several interesting studies with animals demonstrated now a contribution of inflammatory cytokines in the development of pain sensation. In a study of Cunha *et al.* [1991], a dose-dependent hyperalgesia was evoked in the hind paw of rats by an injection of IL-8, which could be lowered with symphatic neurone-blocking agent (guanethidine), dopamnine$_1$ receptor or β-adrenoceptor antagonists [68]. The role of TNF-α in NP mediated pain was demonstrated by different studies. It could be shown that an enhanced response of rats to noxious heat and mechanical stimuli after the application of nucleus pulposus on DRG was due to the TNF-α contained in the NP [69, 70]. The study of Rothman *et al.* [2009] detected elevated mRNA levels of TNF-α, IL-1β and IL-6 in the spinal cord and ipsilateral DRG of rats 1 hour later after applied compression at C7 root for 15 min. Furthermore they observed an increased mechanical allodynia in these treated rats, assessed with a classical method by measuring paw withdrawals of the animal, stimulated with von Frey filaments. When they neutralized TNF-α action with TNF-receptor-1, they observed decreased allodynia in these treated rats and could show that inflammatory cytokines contribute to lumbar radiculopathy [66]. There are several studies demonstrating a role of IL-6 in nociception and pain [64, 71-74]. A study by Arruda *et al.* [1998] measured a central, spinal production of IL-6 in response to a peripheral nerve injury in a rat model [73]. Intrathecal injection of a neutralizing antibody for IL-6 significantly decreased the provoked allodynia in these rats [75]. Another study measured an increased mRNA level of IL-6 in the DRG of mice following constriction nerve injury [76]. Further, Ramirez *et al.* [2008] detected an increased IL-6 receptor level after sciatic nerve crush injury in schwann cells [77]. Several studies have also reported a correlation between nerve ingrowth in diseased IVD and pain, and claim that discogenic back pain is accompanied by a greater innervation into the disc tissue [78-80].

As both symptomatic and asymptomatic degenerated discs can be morphologically very similar, it is reasonable to search for biochemical differences. In relation to an aged and degenerated disc, there are several potential factors which can act as pro-inflammatory and catabolic mediators, thereby evoking pain sensation and supporting IVD degeneration. These will be discussed further in the next chapter.

3. Pro-inflammatory and catabolic mediators

3.1. Pro-inflammatory cascade

3.1.1. TLRs

The initial sensing of infection is mediated by innate pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs). The PRRs recognize broad structural motifs, called the pathogen-associated molecular patterns (PAMPs) which are highly conserved among microbial species but are generally absent from the host, as well as endogenous molecules released from damaged cells, termed damage associated molecular patterns (DAMPs). Sensing of PAMPs and DAMPs lead to an up-regulation of genes involved in inflammatory response [81].

The TLRs are well characterized, consisting of a leucin-rich region at the N-terminus being glycosylated, a trans-membrane helix and a cytoplasmic Toll/IL-1R homology (TIR) domain [82]. In humans there are so far ten different TLRs described. On the plasma membrane located are TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, in the endolysosome TLR3, TLR7, TLR9 and TLR10. They recognize the different PAMPs and DAMPs, and so far it is assumed that LPS is specifically recognized by TLR4, lipoproteins by TLR2, dsRNA by TLR3, and flagellin by TLR5 [83]. Upon ligand binding, the molecule is sandwiched between two extracellular domains, forming an “m”-shaped dimer. TRL2 can build a dimer with TLR1, recognizing triacylated lipopeptides, or TLR6, responding to diacylated lipopeptides, and complexing additionaly with CD14. TLR4 forms a homodimer and complexes with the co-receptor MD-2 as well as CD14 or dimerizes with TLR6 together with the cofactor CD36. LBP (LPS-binding protein) also acts as a cofactor for surface TLRs. It can bind LPS as well as lipoteichoic acid, peptidoglycan and lipopeptides, facilitating the response by transfer of these PAMPs to CD14. CD36 is also a cofactor of TLR2-TLR6 dimerization. Other accessory molecules are TRIL (for TLR3 and TLR4), progranulin (for TLR9), HMGB1 (for TLR9, possibly TLR3 and TLR7) and LL37 (possibly for TLR7 and TLR9) [83]. Upon dimerization of the TLRs, the TIR domain dimers in the cytosol are recognized by adaptor proteins such as MyD88, MAL, TRIF and TRAM. The triggered downstream signalling leads to the

expression of inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-12, antiviral and anti-pathogen proteins and to the initiation of the adaptive immune response [81].
It has been recognized in the recent years that TLRs are not restricted to the initiation of the innate and adaptive immune reaction, but are also important for the maintenance of homeostasis and are thus implicated in chronic inflammatory and autoimmune diseases [84]. PRRs are not only expressed on monocytes/macrophages but also on non immune cells such as synovial fibroblast [85-87], chondrocytes [88, 89] and hepatic cells [90] showing an increased expression of certain TLRs in degeneration or diseases e.g osteoarthritis (OA) [89], rheumatoid arthritis (RA) [86] or playing a role in fibrosis or liver carcinogenesis [90]. Downstream signalling targets of TLRs known today are the NF-κB pathway and MAP kinases [81-83, 91].

3.1.2. NF-κB

Nuclear factor kappa enhancer binding protein (NF-κB) regulates diverse biological processes including immunity, inflammation, cell proliferation, differentiation, apoptosis and tumorigenesis [92-94]. The NF-κB family consists of five protein subunits, p50, p52, p65 (also known as RelA), c-Rel and RelB [95], shuttling between cytoplasm and nucleus either as homodimer or heterodimer. p50 and p52 are produced by proteasomal processing of the precursors p105 and p100. The NF-κB dimers are retained in the cytoplasm by IκB proteins (IκBα, IκBβ, IκBγ, IκBε, IκB–R and Bcl-3). IκB phosphorylation, ubiquitination and proteasomal degradation leads to the release of the NF-κB dimer, which is now free to translocate into the nucleus and to bind to its consensus sequence on the DNA together with co-activators such as p300/CBP. Stimulation of the cell with TNF-α, IL-1β or TLR ligands activate the canonical NF-κB pathway. This includes the IκB kinase (IKK) complex with its two catalytic subunits IKKα and IKKβ, responsible for the phosphorylation of IκB, being NEMO (NF-κB essential modulator, or IKKγ) as a regulatory subunit and p50/p65 as the dimer. In the nucleus, further modification of NF-κB can be phosphorylation, methylation, acetylation and ubiquitination to either enhance or weaken the affinity to the DNA or to the co-activators. Upon activation of transcription of target genes, it also up-regulates IκBα,

which can enter the nucleus and transport NF-κB back to cytoplasm and thereby forming a negative feedback loop. The noncanonical NF-κB pathway includes activation of NF-κB-inducing kinase (NIK) and the IKKα subunit, phosphorylation and subsequent polyubiquitination of p100, proteasomal processed to p52, and translocation of the dimer p52/Rel-B into the nucleus [96].

3.1.3. MAP kinases

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) such as e.g. growth factors, cytokines or oxidative stress, having their signals further transmitted by receptor tyrosine kinase (RTK), cytokine receptors but also G protein-coupled receptors (GPCRs). Upon stimulation, MAP kinases are activated by their upstream kinases, the MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK), specificity mediated by binding to scaffold proteins. MAP kinases then translocate as a dimer into the nucleus and phosphorylate many different proteins. Recent finding also suggest nuclear importing proteins are involved in this step. Among the target proteins of MAP kinases are various transcription factors, controlling early-response genes which regulate various cellular activities such as cell cycle (e.g. c-Fos, c-Jun) and differentiation or immune and inflammatory responses. One of the best characterized pathways is the RTK/Ras/MAP kinase pathway. After ligand binding, the dimerization of RTK leads to the activation of its tyrosine kinase and autophosphorylation. Subsequent binding of adapter proteins activates Ras, a GTPase, by the exchange of GDP to GTP, which in turn then binds Raf (a MAPKKK), also a serine/threonine kinase. Hydrolysis of this GTP releases Raf, which then triggers phosphorylation of MEK (a MAPKK). MEK activates its MAP kinase (ERK1/2) by phosphorylation on threonine and tyrosine residues [97, 98]. In mammals, fourteen MAP kinases have been characterized. Among the MAP kinase family, the conventional one are the extracellular signal-regulated kinase 1/2 (ERK1/2 also named MAP kinase 3 and 1), ERK5 (also named MAP kinase 7), Jun N-terminal kinase (JNK1/2/3 also named MAP kinase 8, 9 and 10) and the four p38 isoforms α, β, γ , δ, (also named MAP kinase 14, 11, 12 and 13, respectively) [97, 99].

Activators and target genes of NF-κB and MAP kinases revealed in the past few years concerning inflammatory and catabolic reaction in IVD diseases are summarized in a publication of Wuertz *et al.* [2008] [100]. For instance, NF-κB pathway in IVD is activated by IL-1β, TNF-α and peroxynitrite, MAP kinases by IL-1β, TNF-α, low oxygen and low osmolarity. As target genes for NF-κB, ADAMTs and MMPs were identified, for MAP kinases collagen, iNOS, MMPs and TIMPs.

3.1.4. Cytokines; the wide variety of IL-6 effects

Cytokines are low-molecular–weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of discrete stimuli. Their secretion is generally short-lived, ranging from a few hours to a few days. These proteins assist in regulating the development of immune effector cells, and some cytokines possess direct effector functions of their own. Cytokines bind to specific receptors on the membrane of target cells, triggering signal-transduction pathways that ultimately alter gene expression in the target cells. Cytokines can mediate biological effects at picomolar concentrations, because their affinities to their receptor are high. The activity of cytokines was first recognized over 50 years ago, have since been classified in 4 different groups based on their structure and function: the hematopoietin family, the interferon family, the chemokine family and the tumor necrosis factor family. Common to all groups are the low molecular weight (among 30 kDa) and a high degree of α-helical structure.

Cytokines are not only required for the regulation of hematopoiesis, proliferation and differentiation, thereby only affecting target cells bearing appropriate receptors, but are also important for the development of cellular and humoral immune responses, the induction of the inflammatory response as well as wound healing. Cytokines often induce the synthesis of other cytokines, resulting in a cascade of activity, culminating in either synergistic or antagonistic effects. IL-6 is known to be secreted by macrophages, fibroblasts and endothelial cells and is described to act in the innate immune system and influences the adaptive immunity by stimulating the proliferation and antibody secretion of B cell lineage. In the hematopoiesis it is released by myeloid progenitor cells and

directs the differentiation to leukocytes, mainly responsible for platelet production. A redundant and pleiotropic effect of IL-6 together with IL-1β and TNF-α is the induction of fever via the prostaglandin synthesis stimulation, the synthesis of acute-phase proteins and the increase of vascular permeability (Goldsby: Immunology). IL-6 has a soluble (sIL-6R) and a membrane bound receptor (gp130). sIL-6R is generated either by proteolysis of membrane bound protein or by alternative splicing. gp130 can consists of an additional subunit, a ligand specific subunit designated IL-6Rα (gp80) [101], but is so far only found on some cells including hepatocytes and some leucocytes [102]. Signalling through its dimerization with gp130 receptor leads to the activation of the JAK/STAT pathway [103]. More and more functions of IL-6 are becoming realized, suggesting that IL-6 not only plays an important role in the acute phase in the immune system, but also in catabolic metabolism, apoptosis and even in mediating pain. A participation of IL-6 in pathophysiology includes autoimmune diseases, osteoporosis, alzheimer's, neoplasia aging and cancer [104].

Studies demonstrating a role for IL-6 in nociception and pain are discussed in the chapter of symptomatic degenerated intervertebral disc. In summary, IL-6 and its receptor were found to be up-regulated in peripheral nerves, DRG and spinal cord during experimental pain. Furthermore, IL-6 increased the responses to thermal and mechanical stimuli and pain in animals, which could be prevented by neutralizing IL-6. Moreover, an elevated IL-6 level has been found in disc tissue of patients with discogenic back pain compared to sciatica patient [60]. It is therefore one of our main aims to reveal the responsible factors involved in regulating IL-6 synthesis in symptomatic degenerated discs.

3.2. The ECM as an inflammatory and catabolic mediator

There has now been recognition that degradation products of the ECM can effect an inflammatory reaction involving specific immune cells (e.g. macrophages, B-cells, T-cells) but also extended to other cells such as e.g. fibroblasts, chondrocytes, smooth muscle cells or malignant cells [105]. Furthermore, several studies could demonstrate a participation of ECM fragments in degenerative and destructive cartilage diseases and thus may play a central role in mediating OA and RA.

To date, the majority of studies investigating the involvement of ECM fragments in disease have been focused on fibronectin fragments. In a study of Forsyth *et al.* [2002] it has been shown that fibronectin fragments, which have been found to be increased in cartilage and synovial fluid of OA and RA patients [106, 107], activate MMP13 in human articular chondrocytes through the MAP kinases p38, ERK and JNK pathway by integrin-mediated signalling [108], claiming that this may play a role in the process for progressive cartilage degradation in arthritis. NF-κB-dependent increases in cytokine and chemokine production [109, 110], as well as NO production in human chondrocytes [111], were detected upon treatment with fibronectin fragments [112, 113]. In macrophages, fibronectin fragments increased secretion of MMP9, MMP12, IL-1, IL-6 and TNF-α [113-116]. Studies from Homandberg *et al.* [1992] demonstrated that fibronectin fragments causes chondrolysis *in vitro* [117] associated with release of catabolic cytokines [109] and severe depletion of cartilage proteoglycans [118] *in vivo* [119]. Furthermore, a study conducted in rabbit IVD cells demonstrated that exposure to fibronectin fragments leads to an up-regulation of MMPs [120].

Such effects are not limited to fibronectin fragments only, but may also extend to other ECM components such as collagen, laminin and elastin. Jennings *et al.* [2001] observed an increased gelatinase activity and an induction of matrix degradation in cultured bovine and human chondrocytes and human cartilage explants when fragmented Col-II was added [121]. Fichter *et al.* [2006] investigated these effects in more detail in a study conducted with bovine articular chondrocytes. They demonstrated a stimulating effect of Col-II fragments on mRNA and protein level for MMP2, MMP3, MMP9 and MMP13 [122]. Data from studies by Guo *et al.* [2009] support these findings where fragments of

Col-II up-regulated proteinases MMP1, MMP3, MMP13 and ADAMTS-5 and decreased proteoglycan content in a cartilage tissue culture model, thereby playing a role in physiologic cartilage damage [123]. Klatt *et al.* [2009] revealed a p38- and NF-κB-dependent increase in MMPs and cytokines upon Col-II treatment of primary human chondrocytes [124].

Also laminin and elastin fragments have been shown to possess inflammatory and catabolic effects. Several studies could demonstrated that laminin fragments induce expression of MMP9 [125-127] and TNF-α in monocytes/macrophages [128]. Damaged elastin for instance is implicated in respiratory diseases [129]. It is reported that elastin fragments in the airway as well as in other organs can cause chemotaxis and cell proliferation [130-134]. Different studies on the biological effect of degraded elastin on cells proclaim an overall inflammatory and remodeling profile [130, 135, 136], as they have been shown to increase cytokine and MMP9 expression in lymphocytes [137]. Elastin fragments are also proposed to be involved in the pathogenesis of COPD [138]. In asthma, an increased neutrophil elastase level has been measured [139-141].

A study conducted in IVD cells by Haschtmann *et al.* [2008] characterized the biological response of isolated IVD fragments to *in vitro* culture condition and observed an increased necrotic and apoptotic cell death combined with a catabolic, pro-inflammatory and chemoattractant gene response [142].

Beside the above discussed proteolytic arised fragments of the ECM such as e.g. fibronectin, collagen, laminin or elastin, it has also been demonstrated that fragments of the polysaccharide hyaluronic acid exhibit important inflammatory and catabolic effects.

3.3. Hyaluronic acid

3.3.1. Structure and function

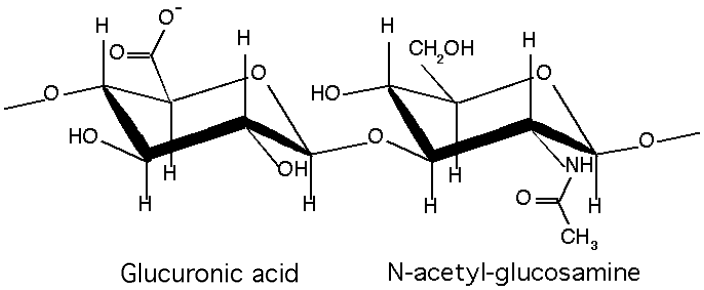


Figure 4. Representation of the viscous HMWHA (upper part) and the repeating glucuronic acid and *N*-acetyl-glucosamine (lower part).

Hyaluronic acid, or hyaluronan (HA) (Figure 4), is generally regarded as an extracellular matrix component being a high molecular polysaccharide composed of repeating *O*-linked disaccharide glucuronic acid and *N*-acetyl-glucosamine (-D-glucuronic acid-β1,3-*N*-acetyl-D-glucosamine-β1,4-]$_n$. At high molecular size, it can reach a weight of 4 x 10^2 - 2 x 10^4 kDa and thereby be composed of 2 x 10^3 - 10^5 sugars. It is known to facilitate cell locomotion, proliferation, differentiation and participates in wound healing. HA dependent pericellular matrix consists of a network of HA filaments, associated with proteoglycans like aggrecan and stabilizing molecules such as link proteins like tenascin, TSG-6, pentraxin, TSP-1 and inter-α-inhibitor. HA is anchored to the cell surface via CD44 [143, 144] or RHAMM (receptor for hyaluronan mediated motility, CD168) [145-148], with CD44 being the most widely expressed HA receptor [149-152] that plays an important role in detaching and locomotion of the cells. CD44 is an ubiquitous

multistructural and multifunctional cell surface adhesion molecule involved in cell-cell and cell-matrix interaction. There exist several isoforms, encoded by a single gene and generated by alternative splicing (extracellular part, membrane proximal region) and post-translational modifications (*N*-glycosylation, *O*-glycosylation, attachment of GAGs, sulfation). The different spliced variants as well the degree of *N*-glycosylation affects the ligand-binding characteristic and can alter the affinity to HA [153-158]. CD44 has one transmembrane spanning region, a N-terminal extracellular domain and a C-terminal cytoplasmic functional tail with several phosphorylation sites that appear to regulate the interaction of CD44 with the cytoskeleton. Additional CD44 ligands include osteopontin, serglycin, collagens, fibronectin and laminin [159-161]. CD44 is implicated in tumor invasion and metastasis. In cancer cells, CD44 is cleaved at the membrane-proximal region, demonstrated to be inhibited in a cell free assay by the tissue inhibitor of metalloproteinase-1 (TIMP-1) [162]. Alteration in CD44 and HA interaction is not only implicated in cancer progression [163-165], but also in RA [166]. CD44 plays a crucial role in inflammation [167]. The soluble form of CD44 (sCD44), which seems to be proteolytically cleaved cell-surface CD44 [162], has been detected to be low in the plasma in immunodeficiency and is increased in malignant diseases, in immune activation and inflammation [168].

RHAMM has also different isoforms by alternative splicing and is described to be a non-integral cell surface hyaluronan receptor and is reported to be part of a multimeric cell surface-bound complex, termed the HA receptor complex (HARC) [169]. The interaction of RHAMM and HA triggers a phosphorylation cascade [170], leading to cell signalling and migration. It has been implicated in cancer progression as evidenced by its abnormal expression on the surface of B and plasma cells of patients with multiple myeloma [171, 172] or advanced prostate cancer diseases [173]. Together with CD44, it forms complexes with ERK and sustains high basal motility in breast cancer cells [174]. It has been shown that RHAMM can compensate CD44 action in inflammation [175] or wound healing [176] and that it is physically linked to CD44 in aggressive breast cancer cell lines [177]. It is reported that RHAMM is poorly expressed in normal human tissues and is increased during wound repair in response to hypoxia and fibrogenic factors such as TGF-β_1. When stimulated with TGF-β_1, RAHMM has been shown to promote cell

locomotion together with HA in a fibroblast cell line. TGF-β_1 even triggers the transcription, synthesis and membrane expression of RHAMM [178].

With its highly charged character, HA participates in the hydration of the tissue and can act as a space filler, lubricant or shock absorber. It is abundantly present e.g. in the ECM of connective, epithelial and neural tissue, the cartilage, the eye, in the fluid of joint capsule and the umbilical cord. It has now also been reported that HA is not restricted to the extracellular milieu, but is also present intracellularly. It was found in rough endoplasmic reticulum membranes, plasma membranes, inside lysosomes, multivesicular bodies, caveolae and nuclei. Nuclear staining was associated with the dense chromatin [179, 180]. Ripellino *et al.* [1988] discovered HA in developing and adult rat brain, showing intense intracellular staining in neurons and astrocytes [181]. HA was localized to proliferating cells [182] and furthermore shown to accumulate in premitotic and mitotic cells, colocalized with microtubules, RHAMM and the mitotic spindle [183]. Studies in breast cancer cells implicate an overexpression of intracellular RHAMM and suggested to substitute it by the name "intracellular hyaluronic acid binding protein" IHABP [184-186]. IHABP is also thought to be linked to the cell surface by either glycosylphosphatidylinositol anchor or by a so far unidentified linker protein [187]. Other recently identified intracellular HA-binding proteins are P32 and CDC37 [179, 188-190].

HA is synthesized on the cytoplasmic surface of the plasma membrane [191] by several HA synthases (HAS) which are glycosyl transferases and transferred to the outside possibly by the multi-drug resistance (MDR) transporter system [192, 193]. It is not sulfated like all other GAGs. In the ECM, HA serves as the backbone for a non-covalently binding to the molecule aggrecan, which is composed of other GAGs such as chondroitine sulfate and keratan sulfate, together promoting tissue hydration and sustaining the function of the NP as a shock absorber.

Three pathways are nowadays known to degrade HA. The local turnover includes binding, internalization and degradation within the cell by different hyaluronidases (Hyals) (Figure 5). It has been shown that IL-1β and TNF-α are able to induce activation of certain Hyals. At tissue level, HA is released, drained into the vasculature and lymphatic, with final steps at liver, kidney and possibly spleen. Scission of HA can also

be achieved by free radicals [194-196] leading to different sizes of fragmented HA (fHA).

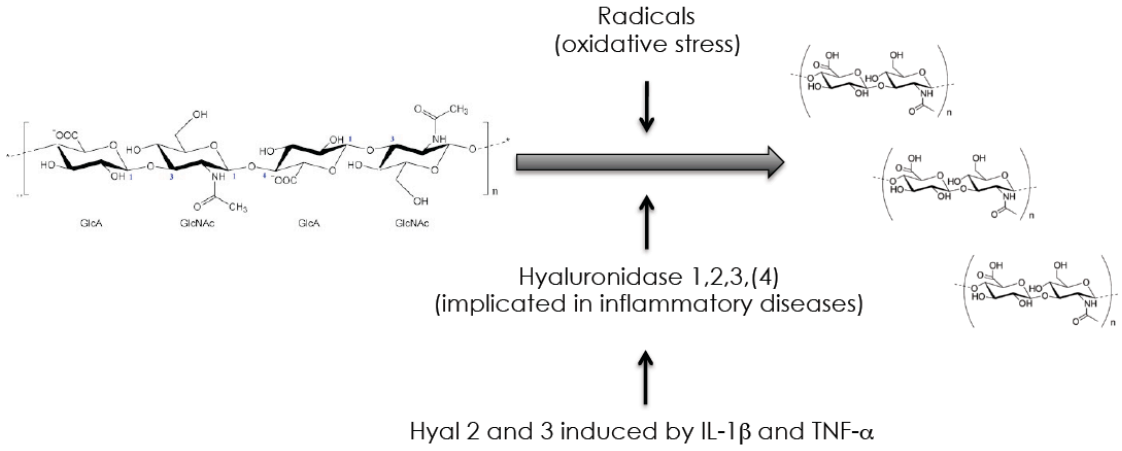


Figure 5. Schematic representation of HMWHA cleavage.

Beside the mentioned structural properties, HA also has a signalling function, depending on HA size. High molecular weight HA (HMWHA) has been described to be anti-angiogenic [197, 198], anti-inflammatory and immunosuppressive [199, 200] whereas fHA can have opposite effects like angiogenic, inflammatory and immunostimulatory and even enhance cancer progression. Depending on cell type, different responses and involved receptors and pathways upon fHA stimulation have been described in the past decades.

3.3.2. Hyaluronic acid fragment effects, engaged receptors and pathways

Depending on the size of the investigated fHA and cell type, different responses involving various receptors and pathways have been described so far upon treatment with fHA. The majority of studies to date have focused on examining the effects of fHA on human articular chondrocytes. Several studies exist regarding the effects of fHA on fibroblasts, macrophages, dendritic cells or human melanoma (see table 2). However, no studies have sought to determine the effects of fHA on IVD cells.

Table 2: Elevated expression level of cytokines, MMPs and NO in fHA treated cells.

Increased mRNA level	Increased protein level	Engaged receptor	Not engaged receptor	General pathway	fHA size	Cell type	Reference
IL-6 IL-1β TNF−α	IL-6 IL-1β TNF−α	TLR4		NF-κB	4-mer	Mouse chondrocytes	Campo *et al.* [2012] [212]
IL-6 IL-1β TNF−α	IL-6 IL-1β TNF−α	TLR4 and CD44		NF-κB	6-mer	Human chondrocytes	Campo *et al.* [2010] [208]
MMP3	MMP3				not determined	Bovine chondrocytes	Schmitz *et al.* [2010] [213]
IL-8 MMP2	IL-8 MMP2	TLR4	 TLR4		4-6-mer	Human melanoma	Voelker *et al.* [2007] [207]
IL-2 MMP2	 MMP2	TLR2	 TLR4	NF−κB	200 kDa (≈1000-mer)	Primary lymphocytes	Scheibner *et al.* [2006] [206]
MIP-1α	MIP-1α	TLR2	TLR4 CD44		200 kDa (≈1000-mer)	Murine alveolar macrophage MH-S	Scheibner *et al.* [2006] [206]
iNOS	iNOS NO				4-8-mer	Human and bovine chondrocytes	Iacob *et al.* [2006] [210]
	NO	CD44			4-8-mer	COS-7	Iacob *et al.* [2006]
iNOS	iNOS NO			JNK p38	500-800 kDa (≈2500-4000-mer)	Murine microglia	Wang *et al.* [2004] [214]
MMP13 MMP9	MMP13 MMP9		TLR4 CD44 TLR4 CD44 RHAMM	NF−κB	not determined	MEF, 3LL	Fieber *et al.* [2004] [202]
MMP13	MMP13		CD44	NF−κB p38	6-mer	Bovine chondrocytes	Ohno *et al.* [2006] [211]
	TNF−α	TLR4	TLR2	p38 ERK NF−κB	4-6-mer	Dentritic cells	Termeer *et al.* [2002] [215]
MME	MME				200 kDa (≈1000-mer)	Mouse alveolar macrophage	Horton *et al.* [1999] [205]
	IL-8 IL-1β TNF−α	 CD44			800 kDa (≈4000-mer)	Human uterine fibroblasts	Kobayashi *et al.* [1997] [216]
IL-8 MIP-1α MIP-1β	 MIP-1α MIP-1β	 CD44			35 kDa (≈175-mer)	Murine alveolar macrophage MH-S	McKee *et al.* [1996] [204]

For instance, in dendritic cells, Termeer *et al.* [2002] showed that fHA (20 μg/ml, 4-6-mer) treatment resulted in an increased TNF-α synthesis with an involvement of p38, ERK and NF-κB. They also monitored an engagement of TLR4 but not TLR2, interestingly together with a downregulation of mRNA for TLR4 and TLR2 level as well as a decreased TLR4 surface-expression upon fHA treatment [201].

Interested in tumor progression, Fieber *et al.* [2004] investigated fHA response in tumor cells. They detected an induced transcription of MMP9 and MMP13 upon fHA (100 μg/ml, size not determined) treatment, but ruled out an involvement of the hyaluronan receptors CD44, RHAMM/IHABP and TLR4. They suggest with their study that HA degradation in tumors or in areas of inflammation might promote invasion or ECM remodeling by activating MMP expression [202]. Sugahara *et al.* [2003] instead demonstrated a participation of CD44 in fHA (5-100 μg/ml, 3-18 ds) treated tumor cells. They revealed that fHA enhanced CD44 cleavage and tumor cell motility [203].

In a murine alveolar macrophages cell line (MH-S), chemokine expression was increased upon treatment with 35 kDa fHA (100 μg/ml) in a study of McKee *et al.* [1996] [204]. Further, Horton *et al.* [1999] measured an increased expression of the metalloproteinase MME (murine metalloelastase) upon treatment with 200 kDa fHA, suggesting that HA fragments may be an important player in inflammatory lung disorders [205]. MH-S was also used in a study by Scheibner *et al.* [2006], in which they observed that the increased expression of macrophage inflammatory protein 1α (MIP-1α) upon fHA exposure was not due to TLR4 or CD44 engagement but TLR2 [206].

TLR4 instead was involved in the response to fHA (50 μg/ml, 4-6 ds) in melanoma cells, leading to an induction of IL-8 but TLR4 independent MMP2 up-regulation [207], thus fHA might contribute to tumor growth and progression.

In primary human chondrocytes, Campo *et al.* [2010] revealed a TLR4 and CD44 engagement in the induced inflammation (increased mRNA and protein level of IL-1β, TNF-α and IL-6) by fHA (10-40 μg/ml, 6-mer) as well as a NF-κB involvement. In contrast to the study by Termeer *et al.* [2002] where a decrease in TLR4 and TLR2 mRNA was observed, Campo *et al.* [2010] measured an increase in TLR4 and CD44 mRNA and protein upon fHA treatment in primary chondrocytes [208]. Further actions

of fHA in chondrocytes observed by other groups were the induction of the cleavage of CD44 (250 μg/ml, size not determined, also seen with 10 ng/ml IL-1β) [209] and the activation of nitric oxide synthase and production of nitric oxide via CD44 (20 μg/ml, 4-8-mer) [210].

In bovine chondrocytes, Ohno *et al.* [2006] demonstrated that fHA (250 μg/ml, 6-mer) enhanced expression of MMP13, mediated in part by CD44. Induction mechanisms were associated with the activation of NF-κB as well as p38 MAP kinase [211]. Recent studies from Campo *et al.* [2012] demonstrated a TLR4 dependent increase in cytokines TNF-α, IL-1β and IL-6 in mouse chondrocytes upon stimulation with fHA (40 μg/ml, 4-mer) with an involvement of the NF-κB pathway [212].

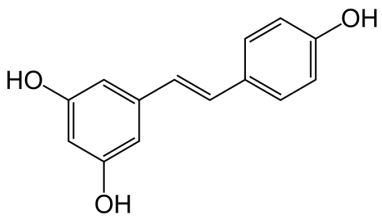
3.3.3. Hyaluronic acid fragments involved in diseases

A study of Eldridge *et al.* [2011] showed increased hyaluronic acid fragmentation and ROS production during pulmonary ischemia in a mouse model. Pretreatment with an antioxidant could reduce ROS as well as fHA production, but did not change Hyal activity, suggesting that ROS released during acute ischemia contributes to HA fragmentation [217]. In a study of Katsumura *et al.* [2004], they checked the effect of AGE, increased in vitreous of diabetic patients, on HA depolymerization. They could measure an increased HA depolimerization *in vitro* by exposure to light and in combination with AGE, claiming that this could provide a new mechanism for diabetic vitreopathy [218]. A current study suggests a contribution of fHA in the skin inflammation disease ACD (allergic contact dermatitis). By inhibiting ROS formation or Hyal activity in a murine keratinocyte cell line or directly in a ACD mouse model, they could measure a decreased fHA production together with a melioration of the inflamed situation [219].

4. Potential biodrugs for the treatment of LBP

The great interest of complementary and alternative therapy by the use of natural products derived from plants for the treatment of diverse diseases such as arthritis, cancer or pain, is demonstrated by the surprising amount of studies investigating healing potential of herbal compounds. Since ancient times, phytopharmaceuticals have been used for the treatment of inflammatory and other disorders. An amazing high number of studies exist for resveratrol, curcuma or triptolide, demonstrating that these substances possess great anti-inflammatory and anti-catabolic effects. We were interested whether these natural herbs also provide beneficial outcome on IVD cells in regard of disc degeneration and as possible treatment substances for discogenic back pain in the near future.

4.1. Resveratrol



The phytoalexin resveratrol (*trans*-3,5,4'-trihydroxystilbene) is found in various plants such as peanuts, berries or grapes, produced by the plant when under attack of pathogens like bacteria or fungi. This polyphenol has attracted great interest in the latest decades as a potential biodrug for the treatment against various diseases such as cancer, type 2 diabetes, osteoarthritis, asthma as well as cardiovascular and neurological diseases, because of its documented anti-inflammatory and antioxidant properties. An amazing number of *in vitro* studies are available, investigating resveratrol for different beneficial inhibitory purposes on different cell types. Consequently, numerous reviews about the effects of resveratrol now exist, describing its various molecular targets and epigenetic changes within the cell, and possible therapeutic applications [220-228]. For instance, the potential of resveratrol as a therapeutic agent for the treatment of osteoarthritis has been

investigated. Zhou *et al.* [2009] showed that resveratrol is able to promote osteoblastic differentiation from pluripotent mesenchymal cells by augmenting the Wnt signalling pathway [229]. They subsequently proposed that resveratrol could be a potential substance for the prophylactic treatment of osteoarthritis. The investigations of Shakibaei *et al.* [2008] in human IL-1β prestimulated chondrocytes provide evidence that resveratrol inhibits the expression of VEGF, MMP3, MMP9 and COX-2. They were furthermore able to show that resveratrol has inhibitory effects on apoptosis and NF-κB, caspase-3 activation and PARP cleavage [230]. By contrast, several cancer-based studies demonstrated enhanced apoptosis induced by elevated resveratrol usage [231-237]. These contrary results could be explained by a study of Howitz *et al.* [2003], who observed that low doses (0.5 μM) of resveratrol stimulated activity of SIRT1, while high doses (>50 μM) had the opposite effect [238]. SIRT1 is a deacetylase and in the active state it decreases the activity and half-life of p53, which is a key player in the regulation of cell cycle arrest and apoptosis.

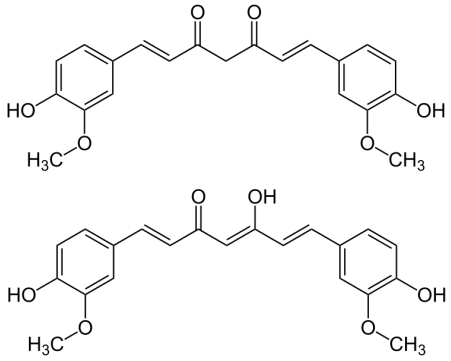
In adipocytes, resveratrol inhibited NF-κB mediated cytokine expression, making this compound interesting for an application in chronic inflammatory adipose tissue and thereby for type 2 diabetes mellitus as well as cardiovascular diseases [239]. Besides having an inhibitory effect on NF-κB activation, resveratrol also influenced MAP kinases and AP-1 [240].

In vivo studies also showed promising effects. In an asthmatic mouse model, resveratrol suppressed IL-4 and IL-5 release by T-helper-2 as well as on eosinophilia and mucus hypersecretion, suggesting an application in the treatment of bronchial asthma [241]. Sebai *et al.* [2009] were able to counteract LPS induced acute phase response in rats with resveratrol, therefore it could be envisaged as a preventing and healing natural compound in endotoxemia induced sepsis [242]. The observed reduced tumorprogression after treatment of a mice xenograft model with resveratrol also hold promise [231].

Resveratrol already showed promising effects on bovine IVD concerning cartilage homeostasis. Li *et al.* [2008] demonstrated an anabolic effect of resveratrol by the increased proteoglycan accumulation in the IVD after treatment with resveratrol [243],

thus supporting the need for further investigations into the use of resveratrol as a biodrug in diseased IVDs.

4.2. Curcuma (Curcumin)



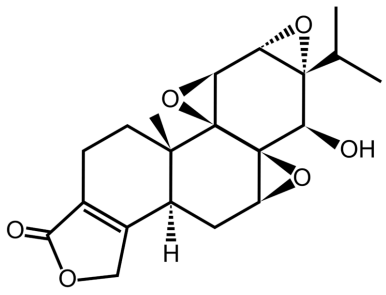
The beneficial effects of curcuma and its component curcumin have been extensively studied over the last three decades and include anticancer, antiviral, antiarthritic, antioxidant and anti-inflammatory properties. For centuries, curcumin has been used in some medical preparation or used as a food-coloring agent. Curcumin is a major component of the turmeric, a yellow spice derived from dried rhizomes of *Curcuma longa*.

In head and neck squamous cell carcinoma, curcumin exhibited anti-cancer properties by suppressing NF-κB activation and stimulating apoptosis via up-regulation of p16 and p53. It showed inhibitory effects on tumor angiogenesis and metastasis via suppression of a variety of growth factors including VEGF, COS-2, MMPs and ICAMs [244]. Yodkeeree *et al.* [2009] demonstrated anti-carcinogenic effects of curcumin in human fibrosarcoma cells. They could show that curcumin exhibited a significant inhibition on cell invasion and the secretion of active MMP2, MMP9 and urokinase plasminogen activator (uPA) [245].

Interested in OA and RA, Buhrmann *et al.* [2010] tested the ability of curcumin to modulate chondrogenic differentiation of mesenchymal stem cells (MSCs). Curcumin showed inhibitory effects on IL-1β prestimulated MSC cells co-cultured with primary chondrocytes. They measured a decreased NF-κB and caspase-3 activation, COX-2 concentration and increased Col-II, cartilage specific proteoglycans as well as β1-

integrin, claiming that curcumin facilitated chondrogenesis of MSC-like progenitor cells *in vivo* [246]. Curcumin also suppressed NF-κB activation in an *in vitro* study performed in human articular chondorcytes by Shakibaei *et al.* [2007]. They also could show an inhibitory effect on COX-2 and MMP9 synthesis as well as a reversion of the IL-1β induced Akt activation and downregulation of Col-II and β1-integrin receptor [247]. These results indicate that curcumin could serve as a potential naturally occurring anti-inflammatory agent for treating OA. In a study of Ma *et al.* [2010], curcumin activated p38, decreased COX-2 concentration and caspase-3 activity in cultured podocytes [248]. In combination with resveratrol, curcumin inhibited NF-κB mediated cytokine (TNF-α, IL-1β, IL-6) expression in adipocytes [239]. *In vivo*, curcumin (200 mg/kg) suppressed mucosal macromolecular leakage, mediated through NF-κB activation, in helicobacter pylori-infected rats, suggesting curcumin for the use as a potent antibacterial agent [249].

4.3. Triptolide



Triptolide is a diterpene lacton and extract of the herb *Tripterygium wilfordii*. It has been used for centuries in Chinese natural medicine as an anti-inflammatory agent for diseases such as RA. Triptolide has not been as extensively investigated as resveratrol or curcuma, but the interest in this natural compound has increased over recent years. There are now also many studies available investigating triptolide for its possible use in inflammatory, autoimmune and immune deficiency diseases or cancer, due to its potent anti-inflammatory, immunosuppressive and tumor suppressive activities.

Interested in anti-inflammatory effects, Matta *et al.* [2009] used triptolide (50 nM) on LPS stimulated macrophages. cDNA microarray analysis revealed that triptolide acts as a selective transcriptional blocker, predominatly affecting genes involved in the immune

response. They could not observe that triptolide was effecting NF-κB activity [250]. Alternatively, Premkumar *et al.* [2010] demonstrated NF-κB involvement as well as TLR and downstream targets such as MyD88 and TRIF in the suppressive effect of triptolide, suggesting that triptolide may have multiple cellular targets contributing to its strong anti-inflammatory and immune suppressive properties [251]. Jonhnson *et al*. [2011] could show that triptolide inhibited proliferation and migration of colon cancer cells. Cell cycle was disturbed by decreased RNA levels of c-myc and A, B, C, and D-type cyclins. Triptolide also decreased expression of VEGF, COX-2 as well as multiple cytokine receptors [252]. In prostate cancer (PC) cells, triptolide inhibited cell growth and induced cell death *in vitro* through caspase activation. *In vivo*, triptolide suppressed xenografted PC-3 tumor growth progression [253].

5. Aim of the thesis

5.1. Purpose

The methods nowadays to treat discogenic back pain are either conservative in nature or dependent on medication, and in the worst cases, invasive through surgical intervention with high risks for the patient as well as high costs for the society. The use of medication suffers in part from the disadvantage of side effects and surgical treatments lack long-term benefits. For a better and more specific targeting of the source of discogenic back pain, it is worth to reveal the biochemical factors and associated signalling pathways leading to this pain sensation. As such, disruption of these signalling pathways through the use of locally administered anti-inflammatory and/or anti-catabolic biodrugs with analgesic effects may be an alternative and less invasive treatment.

5.2. Hypothesis

As high cytokine levels are detected in patients with discogenic back pain and the fact that cytokines may play a role in causing pain sensation directly through nociceptors, we hypothesize that these cytokines from symptomatic IVDs, generated by the disc cells themself, diffuse through the cleft and tears of the disrupted tissue and irritate the nerve endings at the outer part of the AF. We further hypothesize that certain biochemical changes during disc degeneration, such as fragmentation of matrix compounds, are responsible for the innitiation of an inflammatory and catabolic cascade.

5.3. Aims

We are therefore interested

- whether hyaluronic acid fragments (fHA) have the potential to stimulate the synthesis of pro-inflammatory and catabolic mediators in IVD cells *in vitro*.
- which receptors are responsible for regulating IL-6 levels upon fHA treatment. Possible candidates are TLR2, TLR4, CD44 and RHAMM.
- which transduction pathways are involved leading to IL-6 up-regulation by fHA. Possible candidates are NF-κB and MAP kinases (p38, ERK, JNK).
- if bioactive compounds such as resveratrol, curcuma and triptolide exhibit anti-inflammatory and anti-catabolic effects on IVD cells.

RESULTS

6. Overview of published and submitted manuscripts

6.1. Hyaluronic acid fragments enhance the inflammatory and catabolic response in human intervertebral disc cells through modulation of toll-like receptor 2 signaling pathways

Authors: Lilian Quero, Marina Klawitter, Anja Schmaus, Melanie Rothley, Jonathan Sleeman, André N. Tiaden, Juergen Klasen, Norbert Boos, Michael O. Hottiger, Karin Wuertz*, Peter J. Richards*
*authors contributed equally

Journal: Arthritis Res Ther. 2013 Aug 22;**15**(4):R94

Contribution: L. Quero contributed to all the experiments, analysis and statistics.

6.2. Curcuma DMSO extracts and curcumin exhibit an anti-inflammatory and anti-catabolic effect on human intervertebral disc cells, possibly by influencing TLR2 expression and JNK activity

Authors: Marina Klawitter*, Lilian Quero*, Juergen Klasen2 Alexia N. Gloess, Babette Klopprogge, Oliver Hausmann, Norbert Boos, Karin Wuertz
*M. Klawitter and L. Quero have equally contributed to this article.

Journal: J Inflamm (Lond) 2012, **9**(1):29

Contribution: L. Quero contributed to the cell culture experiments, NF-κB assay, RT-PCR measurements as well as analysis and statistic.

6.3. Human MMP28 expression is unresponsive to inflammatory stimuli and does not correlate to the grade of intervertebral disc degeneration

Authors: Marina Klawitter, Lilian Quero, Alessando Bertolo, Marco Mehr, Jivko Stoyanov, Andreas G Nerlich, Juergen Klasen, Nikolaus Aebli, Norbert Boos, Karin Wuertz

Journal: J Negat Results Biomed 2011, **10**:9

Contribution: L. Quero contributed to cell culture experiments, RT-PCR measurements and analysis.

6.4. Triptolide exhibits anti-inflammatory, anti-catabolic as well as anabolic effects and suppresses TLR expression and MAPK activity in IL-1β treated human intervertebral disc cells

Authors: Marina Klawitter, Lilian Quero, Juergen Klasen, Thomas Liebscher, Andreas Nerlich, Norbert Boos, Karin Wuertz

Journal: Eur Spine J (2012) **21** (Suppl 6):S850–S859

Contribution: L. Quero contributed to the cell culture experiments, the RT-PCR measurements as well as analysis and statistic.

6.5. The red wine polyphenol resveratrol shows promising potential for the treatment of nucleus pulposus-mediated pain *in vitro* and *in vivo*

Authors: Karin Wuertz*, Lilian Quero*, Miho Sekiguchi, Marina Klawitter, Andreas Nerlich, Shin-Ichi Konno, Shin-Ichi Kikuchi, Norbert Boos

*K. Wuertz and L. Quero have equally contributed to this article

Journal: Spine (Phila Pa 1976) 2011, **36**(21):E1373-1384

Contribution: L. Quero contributed to all the experiments, analysis and statistics done in this paper except the *in vivo* part.

6.6. Bupivacaine--the deadly friend of intervertebral disc cells?

Authors: Lilian Quero, Marina Klawitter, Andreas G. Nerlich, Massimo Leonardi, Norbert Boos, Karin Wuertz

Journal: Spine J 2011, **11**(1):46-53

Contribution: L. Quero contributed to the cell culture experiments, the RT-PCR measurements as well as analysis and statistics.

Quero et al. Arthritis Research & Therapy 2013, 15:R94
http://arthritis-research.com/content/15/4/R94

arthritis research&therapy

RESEARCH ARTICLE Open Access

Hyaluronic acid fragments enhance the inflammatory and catabolic response in human intervertebral disc cells through modulation of toll-like receptor 2 signalling pathways

Lilian Quero[1,2], Marina Klawitter[3], Anja Schmaus[4], Melanie Rothley[4], Jonathan Sleeman[4,5], André N Tiaden[3], Juergen Klasen[6], Norbert Boos[1,2], Michael O Hottiger[7], Karin Wuertz[1,2,8,9†] and Peter J Richards[3,8*†]

Abstract

Introduction: Intervertebral disc (IVD) degeneration is characterized by extracellular matrix breakdown and is considered to be a primary cause of discogenic back pain. Although increases in pro-inflammatory cytokine levels within degenerating discs are associated with discogenic back pain, the mechanisms leading to their overproduction have not yet been elucidated. As fragmentation of matrix components occurs during IVD degeneration, we assessed the potential involvement of hyaluronic acid fragments (fHAs) in the induction of inflammatory and catabolic mediators.

Methods: Human IVD cells isolated from patient biopsies were stimulated with fHAs (6 to 12 disaccharides) and their effect on cytokine and matrix degrading enzyme production was assessed using quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). The involvement of specific cell surface receptors and signal transduction pathways in mediating the effects of fHAs was tested using small interfering RNA (siRNA) approaches and kinase inhibition assays.

Results: Treatment of IVD cells with fHAs significantly increased mRNA expression levels of interleukin (*IL*)-1β, *IL-6*, *IL-8*, cyclooxygenase (*COX*)-*2*, matrix metalloproteinase (*MMP*)-*1* and -*13*. The stimulatory effects of fHAs on IL-6 protein production were significantly impaired when added to IVD cells in combination with either Toll-like receptor (*TLR*)-*2* siRNA or a TLR2 neutralizing antibody. Furthermore, the ability of fHAs to enhance IL-6 and MMP-3 protein production was found to be dependent on the mitogen-activated protein (MAP) kinase signaling pathway.

Conclusions: These findings suggest that fHAs may have the potential to mediate IVD degeneration and discogenic back pain through activation of the TLR2 signaling pathway in resident IVD cells.

Introduction

Intervertebral disc (IVD) degeneration is considered to be a major contributory factor to the development of discogenic low back pain (LBP), a prevalent and costly musculoskeletal disorder [1,2]. Efforts to develop more effective therapies to combat this condition are hampered by the lack of information relating to the pathophysiological mechanisms responsible for instigating IVD degeneration and the ensuing LBP. There is, however, some evidence suggesting that elevated levels of various pro-inflammatory cytokines within degenerated IVDs may play a decisive role in mediating pain sensation [3-6]. Therefore, a better appreciation of the processes governing cytokine production within degenerated IVDs may help in the development of more effective treatment strategies to combat discogenic LBP.

Breakdown of the IVD extracellular matrix (ECM) is driven by a collection of proteolytic enzymes of which the matrix metalloproteinases (MMPs) and aggrecanases (members of the ADAMTS (A Disintegrin And

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Metalloproteinase with Thrombospondin Motifs) family) have been the most extensively studied [7-10]. These have the potential to degrade numerous matrix components as well as to give rise to a variety of reactive fragment species, which themselves may further act to stimulate and activate IVD cells. This is made evident by findings from our own studies, and from others, where proteolytic fragments of fibronectin and type II collagen have been shown to induce MMP expression in human IVD cells [11-14]. In addition to proteins and proteoglycans, numerous glycosaminoglycans (GAGs) also exist within the IVD, and include hyaluronic acid (HA), chondroitin sulfate and keratan sulfate, although only HA exists in the form of a free GAG [15]. Among these, HA has received significant attention due to the stimulatory nature of its degradation products on various cell types.

HA is a polymer composed of repeating disaccharide units comprised of D-glucuronic acid and D-N-acetylglucosamine. Whilst existing as a high molecular weight (HMW) polymer (>10^6 kDa) under normal conditions, HA can become degraded in response to various pathogenic events resulting in the generation of low molecular weight (LMW) fragments (fHAs) [16]. This may be brought about through the actions of various enzymes, such as hyaluronidases [17], as well as by exposure to non-enzymatic mediators, including reactive oxygen species (ROS) [18]. More specifically, pro-inflammatory agents, such as IL-1β, have been shown to induce the release and fragmentation of HA from cartilage explants [19]. This may be of particular relevance to the development of degenerative disc disease, where reductions in GAG content together with increases in IL-1β are wholly evident in degenerated IVDs [20,21]. Although there is currently no evidence confirming the presence of fHAs within disc tissue, it may be reasonable to assume that the sequence of catabolic and inflammatory events within the degenerating disc could provide an environment conducive to the production of fHAs. However, the potential involvement of such fragments in the pathogenesis of IVD degeneration has not yet been considered. Certainly, fHAs have the capacity to invoke both an inflammatory response as well as induce synthesis of tissue degrading enzymes when added to chondrocytes *in vitro* [22-25]. These effects are mediated through HA cell surface receptors CD44 and/or toll-like receptor (TLR)-4, with subsequent activation of NF-κB [24,25]. The receptor for hyaluronan-mediated motility (RHAMM, CD168) may also represent an additional means through which fHAs could mediate their stimulatory effects [26]. However, no studies have yet sought to investigate the influence of fHAs on the inflammatory and catabolic response in human IVD cells, and to assess their possible mode of action.

In the current report, we have set out to investigate the *in vitro* effects of fHAs on human IVD cells isolated from the discs of patients undergoing spine surgery. Small fHAs ranging in size from 12 to 24 mer were incubated with IVD cells and their influence on inflammatory and catabolic processes evaluated. Furthermore, studies were conducted in an attempt to identify the signalling pathways responsible for mediating the effects of fHAs. Our results clearly demonstrate that fHAs enhance both the pro-inflammatory and catabolic response in IVD cells, being mediated primarily through the TLR2 signaling pathway. These findings may be considered of significant clinical importance, based on the fact that increases in pro-inflammatory cytokine and MMP production are main features of IVD degeneration.

Materials and methods

Isolation and culture of IVD cells

Human IVD tissue was obtained from patients undergoing spinal surgery for symptomatic degenerative disc disease, disc herniation or spinal trauma following informed consent in accordance with the Ethics Committee of the Canton of Zurich (carried out at University Hospital Balgrist, Zurich, Switzerland) (Table 1) and

Table 1 Details of patients used in the study.

Patient	Pathology	Severity Grade[a]	Disc Level
1	DH	4	L4/5
2	SD	5	L4/5
3	DH	5	L4/5
4	DH	5	C5/6
5	DH	5	L5/S1
6	DH	4	L4/5
7	DH	3	L5/S1
8	DH	5	L5/S1
9	DH	4	L4/5
10	DH	4	L4/5
11	DH	5	L4/5
12	DH	4	L4/5
13	DH	3	L2/3
14	DH	4	L5/S1
15	DH	4	L5/S1
16	DH	5	L5/S1
17	DH	3	L5/S1
18	DH	5	L4/5
19	DH	4	L5/S1
20	DH	4	C6/7
21	DH	4	L4/5
22	DH	5	L5/S1

[a] The degree of IVD degeneration in patients was assessed prior to surgical intervention by magnetic resonance imaging (MRI) using a 5-level grading system based on Pfirrmann's classification of disc degeneration.

C, cervical; DH, disc herniation; F, female; L, lumber; M, male; S, sacral; SD, segment degeneration

IVD cells isolated and cultured as previously described [11]. Cells were used for experiments at passages 2 to 3.

Preparation of HA fragments

Hyaluronic acid oligomers of 6 to 12 disaccharide units in length were prepared as previously described [27]. Ultrapure HMW HA (Healon 5), kindly provided by Amo (Ettlingen, Germany), was dissolved at 5 mg/ml in 0.3 M sodium phosphate buffer, pH 5.3, sonified and subsequently enzymatically digested with 200 U/ml bovine testis hyaluronidase (Sigma-Aldrich, Seelze, Germany) for six hours at 37°C. The resulting fragments were separated on a Bio Gel P10 column (3.5 × 115 cm) (BioRad, Munich, Germany) and 3 ml fractions collected. The concentration of HA in the fractions was determined by measuring the absorbance at 210 nm with reference to standards. The fractions were tested for endotoxin contamination using the Limulus Amebocyte Lysate (LAL)-assay kit (Lonza, Verviers, Belgium) according to the manufacturer's instructions. In all cases, endotoxin levels were below detection limits.

For determination of HA fragment size, fluorophore-assisted carbohydrate electrophoresis (FACE) analysis of 7-amino-1,3-naphthalenedisulfonic acid (ANDS)-labeled fragments was performed. Briefly, samples were dried and resuspended in 5 μl 0.15 M ANDS (in 0.15% Acetic Acid) and 5 μl 1 M $NaCNBH_4$ (in DMSO) (both Sigma-Aldrich). After 16 hours at 37°C the samples were dried and resuspended in 20% glycerine. Samples were separated on a 30% polyacrylamide gel at 15 mA. Bands were visualized in the gel by UV illumination. The size of the oligosaccharides in the fractions was determined by comparing the bands with similarly labeled, commercially available HA fragments of defined sizes (Sigma-Aldrich) (Figure 1).

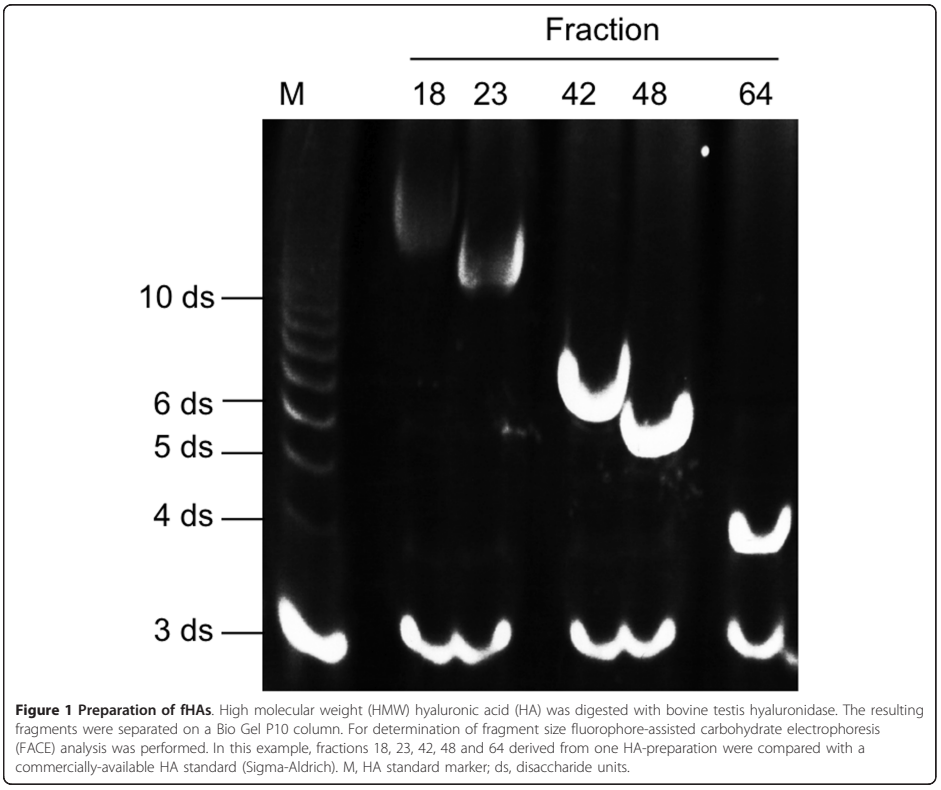

Figure 1 Preparation of fHAs. High molecular weight (HMW) hyaluronic acid (HA) was digested with bovine testis hyaluronidase. The resulting fragments were separated on a Bio Gel P10 column. For determination of fragment size fluorophore-assisted carbohydrate electrophoresis (FACE) analysis was performed. In this example, fractions 18, 23, 42, 48 and 64 derived from one HA-preparation were compared with a commercially-available HA standard (Sigma-Aldrich). M, HA standard marker; ds, disaccharide units.

Gene expression profile in IVD cells treated with fHAs

IVD cells (1×10^6) were cultured in 150 cm^2 flasks and starved in serum-free medium for 2 h prior to stimulation. Cells were incubated in medium alone or medium supplemented with fHAs (5 or 20 μg/ml) for up to 18 hours. Total RNA was isolated from IVD cells using the PureLink RNA Mini kit (Life Technologies, Zug, Switzerland) according to the manufacturer's recommendation (Life Technologies), then 1 μg total RNA was reverse-transcribed using Superscript II (Life Technologies). Quantification of mRNA expression was performed on the StepOnePlus Real-Time PCR System (Life Technologies) using the TaqMan Gene Expression Assays (Life Technologies) specific for *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, *MMP-1*, *-2*, *-3*, *-9*, *-13*, *COX-2*, *ADAMTS4* and *ADAMTS5* (Table 2). Values were normalized to TATA-Box binding protein (*TBP*) mRNA levels and presented as either $2^{-\Delta CT}$ or as fold change as compared to untreated cells according to the $2^{-\Delta\Delta CT}$ method where stated.

Stimulation of IL-6 production in IVD cells

IVD cells (1.3 $x10^5$) were cultured in 12-well plates and starved in serum-free medium for 2 h prior to stimulation. Cells were incubated in medium alone or medium supplemented with fHAs (20 μg/ml), Pam3CysSerLys4 (Pam3CSK4) (25 ng/ml) (LabForce, Nunningen, Switzerland), IL-1β (5 ng/ml) (Peprotech, London, UK) or lipopolysaccharide (LPS) (25 ng/ml) (**LuBioScience**, Luzern, Switzerland) for up to 18 hours. Culture supernatants were harvested for further analysis using a specific IL-6 ELISA (BD Biosciences, Allschwil, Switzerland).

The effect of gene silencing on fHA-mediated IL-6 production in IVD cells

Specific knock down of *TLR2*, *TLR4*, *CD44* and *RHAMM* expression was performed with small interfering (si)RNA oligos (Qiagen, Hombrechtikon, Switzerland). Human IVD cells (1.3×10^5 cells) were transfected with 10 or 20 nM of siRNA specific for *TLR2* (SI00050036), *TLR4* (SI04951149), *CD44* (SI00299705), or *RHAMM* (SI04 435347), or negative control siRNA (SI03650325) using lipofectamine RNAiMAX (Life Technologies) in 12-well plates. Following transfection, cells were incubated with fresh growth medium (without antibiotics) and incubated for 24 hours at 37°C, 5% CO_2. Cells were then stimulated with fHAs (20 μg/ml) for 18 hours and culture supernatants harvested for further analysis using a specific IL-6 ELISA (BD Biosciences).

Table 2 TaqMan gene expression assays used for qRT-PCR.

Target gene	Assay ID
TATA box binding protein (*TBP*)	Hs00427620_m1
Interleukin 1 β (*IL-1β*)	Hs00174097_m1
Interleukin 6 (*IL-6*)	Hs00174131_m1
Interleukin 8 (*IL-8*)	Hs00174103_m1
Tumor Necrosis Factor α (*TNFα*)	Hs00174128_m1
Matrix metalloproteinase 1 (*MMP-1*)	Hs00233958_m1
Matrix metalloproteinase 2 (*MMP-2*)	Hs01548724_m1
Matrix metalloproteinase 3 (*MMP-3*)	Hs00968308_m1
Matrix metalloproteinase 9 (*MMP-9*)	Hs00957555_m1
Matrix metalloproteinase 13 (*MMP-13*)	Hs00233992_m1
Cyclooxygenase 2 (*COX-2*)	Hs00153133_m1
Aggrecanase 1 (*ADAMTS4, AD4*)	Hs00943031_g1
Aggrecanase 2 (*ADAMTS5, AD5*)	Hs00199841_m1

The effect of TLR2 inhibition on fHA-mediated IL-6 production in IVD cells

IVD cells (1.3×10^5) were cultured in 12-well plates and starved in serum-free medium for 2 h prior to stimulation. Cells were then pre-incubated for one hour with either an affinity purified polyclonal rat anti-human TLR2 neutralizing antibody (final concentration 5 μg/ml) (LabForce, Switzerland) or an isotype matched IgG control (Lucerna-Chem, Luzern, Switzerland). Cells were then stimulated with fHAs (20 μg/ml) or Pam3CSK4 (25 ng/ml) for 18 hours and culture supernatants harvested for further analysis using a specific IL-6 ELISA (BD Biosciences).

The role of NF-κB in fHA-dependent IVD cell activation

IVD cells (3×10^5) were cultured in six-well plates and starved in serum-free medium for 2 h prior to stimulation. Cells were then treated for up to one hour with either fHAs (20 μg/ml) or IL-1β (5 ng/ml). For the detection of NF-κB (p65) by immunofluorescence, cells were fixed with ice cold methanol (-20°C) for 10 minutes at selected time points, blocked for 10 minutes with PBS containing 1% BSA (Sigma) and 0.1% Triton-X100 (Sigma), and incubated with polyclonal rabbit anti-NF-κB (p65) (Santa Cruz Biotechnologies, Heidelberg, Germany) (1:200) for one hour at room temperature. NF-κB (p65) was detected using goat anti-rabbit Cy2 (Jackson ImmunoResearch, Newmarket, Suffolk, UK) (1:200) and visualized by fluorescence microscopy. For Western blot analysis, cells were first washed with buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 5 mM DTT with freshly added protease inhibitor cocktail (Sigma-Aldrich) and then lysed with 0.1% NP-40 for five minutes on ice. Nuclear pellets were harvested after centrifugation at 10,000 rpm for 5 minutes at 4°C, and lysed for 20 minutes in buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM PMSF and 5 mM DTT. Protein concentrations were determined using the Bradford Assay (BioRad) and equal amounts loaded onto 12% SDS-PAGE gels. Protein was subsequently electroblotted onto PVDF membranes and incubated with nonfat dry milk (5%), 50 mM

Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20 (TBST) for one hour at room temperature. Membranes were then incubated for 2 hours at room temperature with either anti- NF-κB (p65) (1:200) or anti-PARP1 (1:1,000) (both from LabForce, Switzerland). After washing in TBST three times for five minutes each, membranes were incubated with an appropriate HRP-conjugated secondary antibody for one hour at room temperature. Following a further washing step, peroxidase activity was detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Lausanne, Switzerland). NF-κB (p65) binding activity was measured in nuclear extracts from IVD cells using the NF-κB (p65) Transcription Factor Assay according to the manufacturer's recommendations (Cayman, Tallinn, Estonia). All absorbance measurements were carried out at 655 nm.

Role of MAP kinases in mediating the effects of fHAs in IVD cells

Cultured IVD cells were treated for 15 minutes with fHAs (20 μg/ml), IL-1β (5 ng/ml), LPS (25 ng/ml) or left untreated and whole protein cell extracts isolated following lysis in buffer containing 50 mM HEPES (pH 7.5), 450 mM NaCl, 15% glycerol, 2 mM EDTA, 1 mM PMSF and a freshly added protease inhibitor cocktail (Sigma-Aldrich). Protein was harvested following centrifugation at 14,000 rpm for 30 minutes and equal amounts loaded onto 12% SDS-PAGE gels and transferred to PVDF. Membranes were then incubated for two hours with either rabbit anti-p38 (1:1,000), rabbit anti-phospho-p38 (Thr180/Tyr182) (1:1,000), rabbit anti-p44/44 (1:1,000), rabbit anti-phospho-p44/42 (1:1,000), rabbit anti-SAPK/JNK (1:1,000) or rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:200) (all from Cell Signaling Technology, Allschwil, Switzerland) and further analysed as described above. The functional role of MAP kinases in mediating the effects of fHAs was investigated using MAP kinase inhibitors. IVD cells were treated with fHA (20 μg/ml) alone (Control) or in combination with MAP kinase inhibitors (10 μM) specific for p38 (SB203580), ERK 1/2 (PD98059) or SAPK/JNK (SP600125) and culture supernatants harvested for further analysis using ELISAs specific for IL-6 (BD Biosciences) or MMP-3 (R and D Systems, Abingdon, UK) according to the manufacturer's protocol.

Statistical analysis

All statistical analyses were carried out using SPSS19.0 (SPSS Inc., Chicago, IL, USA). Data were first assessed for normality of distribution using the Kolmogorov-Smirnov test. Parametric analysis of normally distributed data was performed using the two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* tests for multiple group comparisons. Non-parametric data were analyzed using the Kruskal-Wallis one-way analysis of variance for multiple group comparisons followed by the Mann-Whitney U test for comparisons between two groups. A *P*-value of <0.05 was considered statistically significant. All data were expressed as mean ± standard deviation (S.D.).

Results

Effect of fHAs on the expression of inflammatory and catabolic genes in IVD cells

Initial experiments were undertaken in order to assess the effects of fHAs on the expression of a range of inflammatory and catabolic genes in cultured IVD cells. Treatment of IVD cells was carried out for up to 18 hours using concentrations of fHAs based on previous studies [24,28]. Stimulation of cells with fHAs at either 5 or 20 μg/ml resulted in significant alterations in several of the genes analysed (Figure 2). The most noticeable effects were observed in cells treated with the higher dose of fHAs (20 μg/ml), where significant increases in expression levels were measured for *IL-1β* (P <0.01), *IL-6* (P <0.05), *IL-8* (P <0.01), *MMP-1* (P <0.01), *MMP-13* (P <0.05) and *COX-2* (P <0.01). All subsequent experiments therefore involved the use of fHAs at 20 μg/ml.

The stimulatory effect of fHAs on IVD cells is dependent on functionally active TLR2

Of the genes identified as being regulated by fHAs, *IL-6* was considered an appropriate candidate for further investigations based on its roles as both a pro-inflammatory cytokine and also as a mediator of pain [3].

Stimulation of IVD cells with fHAs induced a significant increase in IL-6 protein production (3.5 ± 1.5 ng/ml; P <0.01) as compared to untreated cells (Figure 3A), as did other well-known instigators of IL-6 production, including toll-like receptor activators Pam3CSK4 (6.0 ± 3 ng/ml) (Figure 3B) and LPS (10.8 ± 7.85 ng/ml) (Figure 3C), as well as the pro-inflammatory cytokine IL-1β (120 ± 36 ng/ml) (Figure 3D).

An siRNA approach was then used to target genes encoding the cell surface receptors *TLR2*, *TLR4*, *CD44* and *RHAMM*, with the aim of identifying potential receptors involved in engaging fHAs. Knockdown efficiency was confirmed in IVD cells after 24 hours using qRT-PCR (Figure 4A). TLR2 and TLR4 were selected for further evaluation of functional loss of receptor activity. Confirmation of efficient and comparable TLR2 and TLR4 loss-of-function was substantiated in siRNA-treated cells through examination of their ability to express IL-6 following incubation with TLR ligands Pam3CSK4 (Figure 4B) and LPS (Figure 4C). fHA-dependent IL-6 production by IVD cells was also significantly reduced following TLR2 loss-of-function (Figure 4D), although no

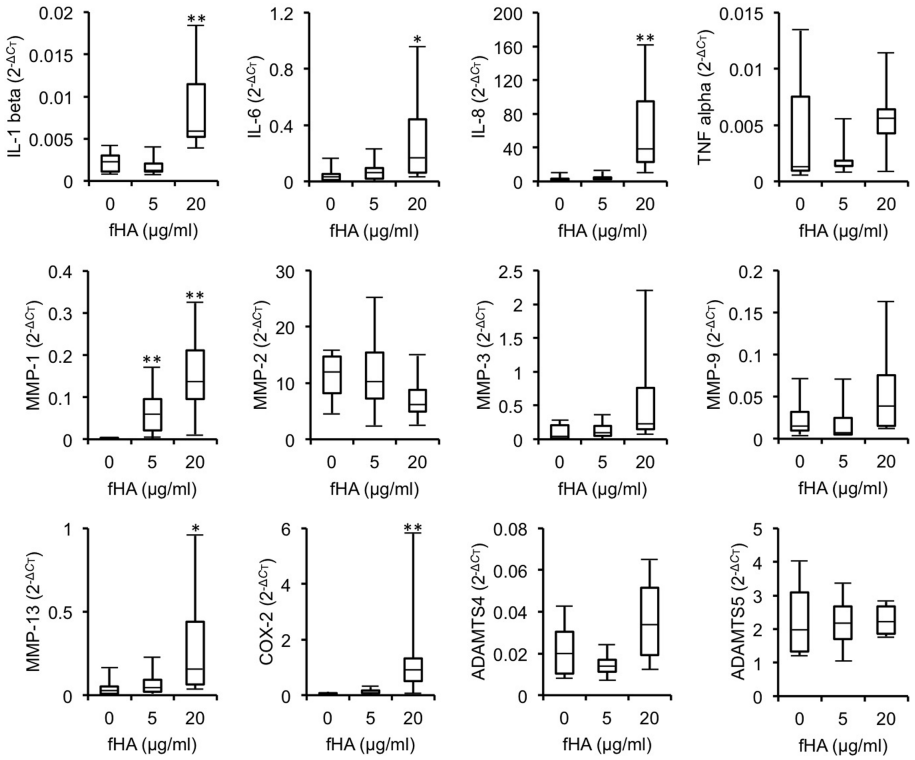

Figure 2 Gene expression profile in IVD cells treated with fHAs. Human IVD cells were incubated for 18 hours with hyaluronic acid fragments (fHAs) at either 5 or 20 μg/ml (n = 4 to 6) and RNA harvested for analysis by qRT-PCR. Values were normalized to TATA-Box binding protein (*TBP*) mRNA and expressed as $2^{-\Delta CT}$. Statistical analysis was performed using the Kruskal-Wallis one-way analysis of variance for multiple group comparisons followed by the Mann-Whitney U test for comparisons between two groups, * P <0.05, ** P <0.01 as compared to untreated IVD cells. *IL*, interleukin; *TNF*, tumor necrosis factor; *MMP*, matrix metalloproteinase; *COX*, cyclooxygenase; *ADAMTS*, A Disintegrin And Metalloproteinase with Thrombospondin Motifs.

significant reductions in IL-6 production were observed in cells in which TLR4, CD44 or RHAMM had been knocked down.

Further confirmation of TLR2's involvement in the activation of IVD cells by fHAs was demonstrated in antibody-mediated neutralization studies. Initial studies confirmed the effectiveness of the polyclonal anti-TLR2 antibody to neutralize TLR2 activity as demonstrated by its ability to suppress Pam3CSK4-dependent IL-6 production as compared to a non-specific IgG control antibody (Figure 5A). Similarly, antibody-mediated TLR2 inactivation also significantly reduced (P <0.05) the stimulatory effects of fHAs on IL-6 production by IVD cells (Figure 5B).

The role of NF-κB in fHA-mediated IVD activation

Activation of NF-κB is considered to be a primary means through which fHAs mediate their stimulatory effects in chondrocytes [24,25]. We, therefore, carried out a series of experiments to investigate whether fHAs could also induce NF-κB activation in human IVD cells.

We were unable to observe any evidence of NF-κB activation in IVD cells following stimulation with fHAs (20 μg/ml). This was clearly demonstrated by the lack of any increase in nuclear p65 as determined by both immunofluorescence staining (Figure 6A) and also Western blot analysis (Figure 6B) as compared to untreated cells. Furthermore, nuclear extracts harvested from IVD

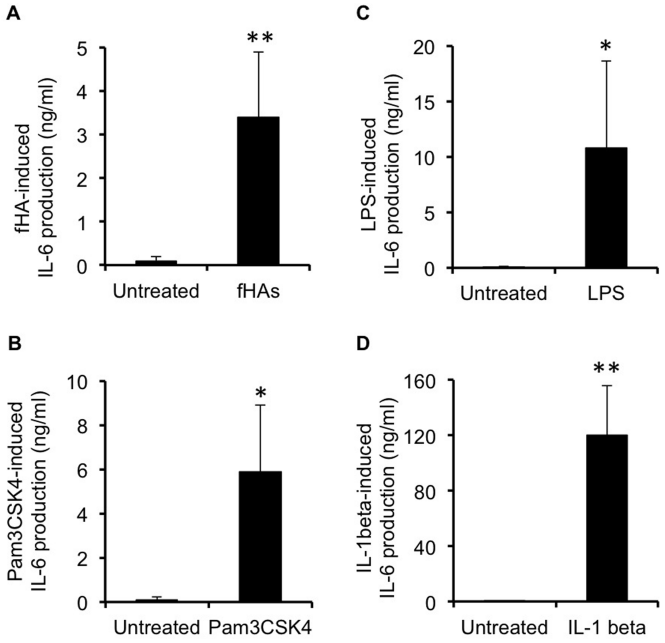

Figure 3 Stimulation of IL-6 production in IVD cells. Human intervertebral disc (IVD) cells were incubated for 18 hours with either: hyaluronic acid fragments (fHAs) (20 μg/ml) (n = 4) (**A**); Pam3CSK4 (25 ng/ml) (n = 4) (**B**); lipopolysaccharide (LPS) (25 ng/ml) (n = 5) (**C**); or interleukin (IL)-1β (5 ng/ml) (n = 5) (**D**), and IL-6 protein levels determined in supernatants using a specific ELISA. In all cases, analyses were performed in triplicate and values expressed as mean ± S.D. Statistical analysis was performed using the Student's t-test, * P <0.05, ** P <0.01 as compared to untreated IVD cells.

cells treated with fHAs demonstrated no significant increase in NF-κB (p65) DNA binding activity when tested using a specific transcription factor assay (Figure 6C). This was in direct contrast to cells treated with IL-1β, where obvious increases in nuclear p65 were evident in all of the performed analyses.

Activation of IVD cells by fHAs is dependent on MAP kinase signalling pathways

Based on the fact that TLR2 signalling also partly relies on the MAP kinase pathway [29], we next investigated whether fHAs had the capacity to activate various MAP kinases in IVD cells. Western blot analysis revealed a marked increase in the level of phosphorylated c-Jun-N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (Figure 7A). In addition, we also saw a noticeable increase in the level of phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (p44/p42) in cells treated with fHAs (Figure 7B). However, by comparison, only marginal increases in the level of p38 MAP kinase phosphorylation were observed in response to fHAs (Figure 7C).

In order to further clarify the potential involvement of MAP kinase activity in regulating fHA-dependent IVD cell activation, we performed additional experiments utilizing specific MAP kinase inhibitors. The stimulatory effects of fHAs on IL-6 production by IVD cells were significantly reduced following pre-exposure of the cells to either ERK inhibitor PD98059 (P <0.05) or JNK inhibitor SP600125 (P <0.01) (Figure 7D). The p38 MAP kinase inhibitor, SB203580, had only a weak inhibitory effect on fHA-dependent IL-6 production, which did not reach statistical significance (P = 0.27). We also investigated the ability of MAP kinase inhibitors to affect fHA-induced MMP production in IVD cells. As with IL-6, MMP-3 protein production was up-regulated by over four-fold (P <0.01) in IVD cells treated with fHAs (Figure 7E) and was significantly reduced following inhibition of JNK activity (P <0.01).

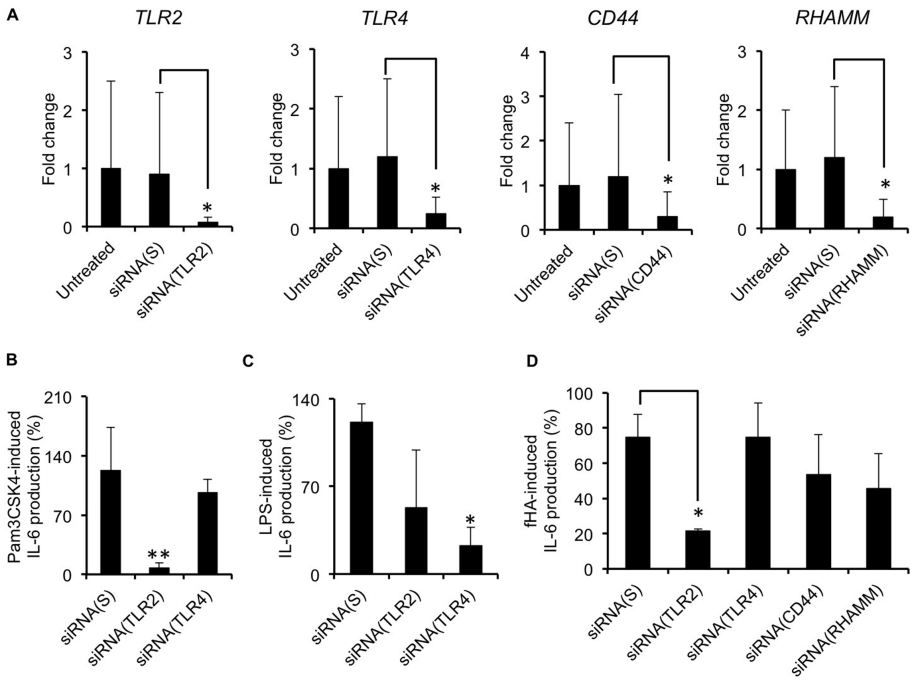

Figure 4 The effect of gene silencing on fHA-mediated IL-6 production in IVD cells. **A**) siRNA-mediated knockdown of genes Toll like receptor (*TLR*)*2*, *TLR4*, *CD44* and *RHAMM* was confirmed after 30 hours in intervertebral disc (IVD) cells by qRT-PCR ($n = 4$). In each case, gene expression was calculated as fold change as compared to untreated cells. The use of a non-specific scrambled siRNA (siRNA (S)), confirmed specificity of gene knockdown. **B, C**) Interleukin (IL)-6 protein production by Pam3CSK4- (25 ng/ml) ($n = 4$) (**B**) and lipopolysaccharide (LPS)- (25 ng/ml) ($n = 3$). (**C**) stimulated IVD cells following gene knockdown of *TLR2* or *TLR4* respectively, as determined by IL-6 ELISA. (**D**) hyaluronic acid fragment (fHA)-treated (20 μg/ml) IVD cells following gene knockdown as determined by IL-6 ELISA ($n = 4$). IL-6 protein levels are represented as a percentage of those measured for untreated cells. In all cases, analyses were performed in triplicate and values expressed as mean ± S.D. Statistical analysis was performed using the Student's *t*-test, * $P < 0.01$.

Discussion

The primary source of discogenic back pain has been debated over the years, although there is now a growing body of evidence suggesting a close causal relationship between pro-inflammatory cytokine expression and the development of pain within the degenerated IVD [3,10,30]. Certainly, pro-inflammatory mediators have the capacity to provoke pain sensation [4-6], although it remains unclear as to which processes are responsible for initiating the ensuing cascade of cytokines in diseased discs. Considering the fact that discogenic back pain occurs primarily in degenerated discs, it is highly likely that the processes governing disc degeneration also play a role in instigating pro-inflammatory cytokine production.

Degradation of the ECM through catabolic processes can result in the generation of a variety of matrix protein fragments with the potential to influence cellular behavior in numerous tissue types, usually with a detrimental outcome [9,11,12,31-33]. Furthermore, fragments generated from HA have the potential to induce a number of pro-inflammatory responses as evidenced by their ability to up-regulate chemokines, cytokines and matrix degrading enzymes in several different cell types, including chondrocytes [22-27,34-36]. In the present report, we have extended these observations to include human IVD cells, where fHA-mediated stimulation was shown to significantly enhance the expression of pro-inflammatory cytokines *IL-1β*, *IL-6* and *IL-8*. Moreover, fHAs were also found to stimulate the expression of certain

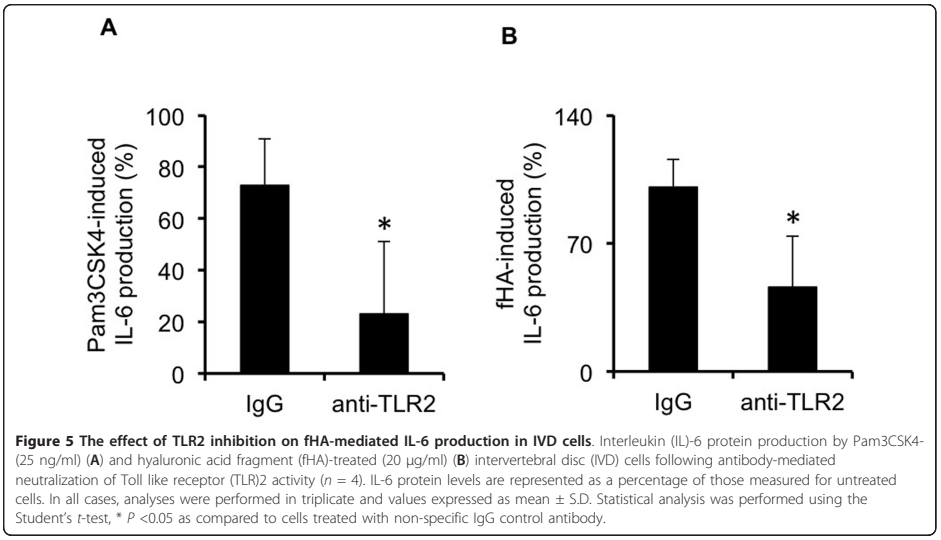

Figure 5 The effect of TLR2 inhibition on fHA-mediated IL-6 production in IVD cells. Interleukin (IL)-6 protein production by Pam3CSK4- (25 ng/ml) (**A**) and hyaluronic acid fragment (fHA)-treated (20 μg/ml) (**B**) intervertebral disc (IVD) cells following antibody-mediated neutralization of Toll like receptor (TLR)2 activity ($n = 4$). IL-6 protein levels are represented as a percentage of those measured for untreated cells. In all cases, analyses were performed in triplicate and values expressed as mean ± S.D. Statistical analysis was performed using the Student's *t*-test, * $P < 0.05$ as compared to cells treated with non-specific IgG control antibody.

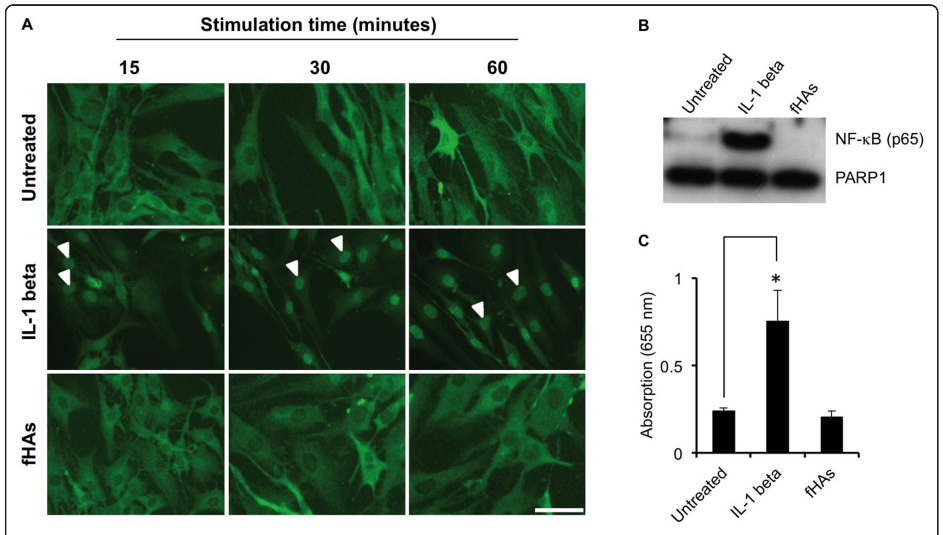

Figure 6 The role of NF-κB in fHA-dependent IVD cell activation. (**A**) Immunofluorescence staining of intervertebral disc (IVD) cells for NF-κB (p65) (green) following treatment with either hyaluronic acid fragments (fHAs) (20 μg/ml) or interleukin (IL)-1β (5 ng/ml) at selected time points. Nuclear NF-κB (p65) is indicated by arrowheads. Untreated IVD cells served as a control. Scale bar = 50 μm. (**B**) Western blot analysis of nuclear extracts from untreated IVD cells or cells treated for 1 hour with either fHAs (20 μg/ml) or IL-1β (5 ng/ml). Levels of PARP1 protein served as a loading control. (**C**) NF-κB (p65) DNA binding activity in nuclear extracts from untreated IVD cells, or cells treated for one hour with IL-1β or fHAs (20 μg/ml) ($n = 3$). In all cases, analyses were performed in triplicate and values expressed as mean ± S.D. Statistical analysis was performed using the Student's *t*-test, * $P < 0.01$ as compared to untreated cells.

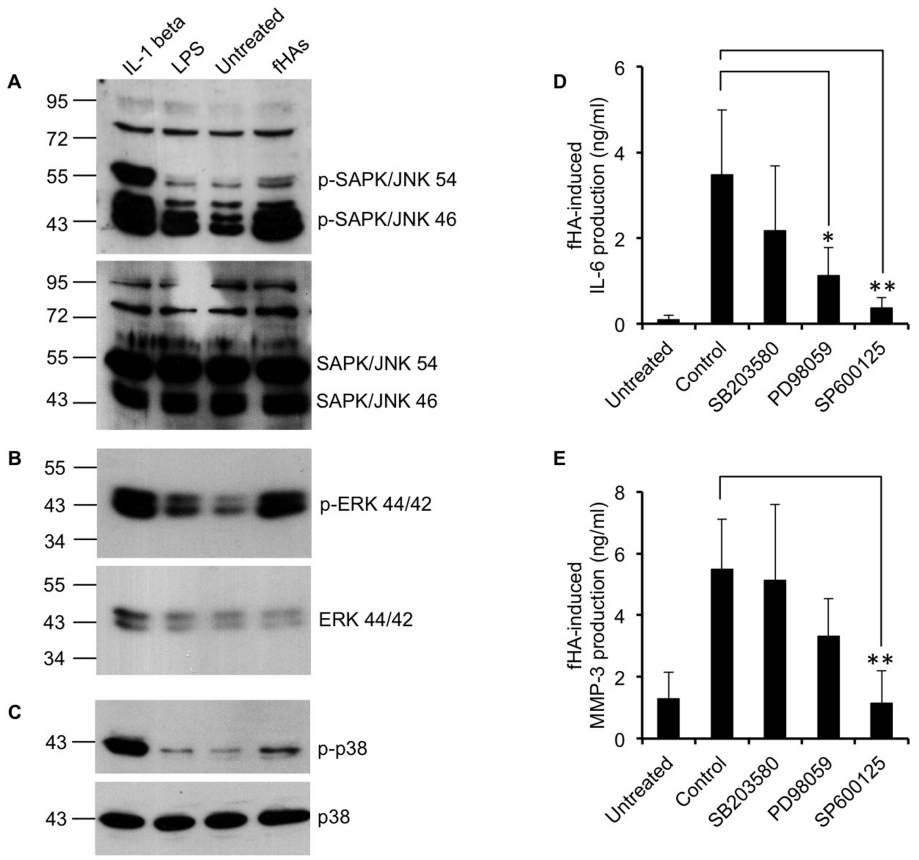

Figure 7 Role of MAP kinases in mediating the effects of fHAs in IVD cells. Cultured intervertebral disc (IVD) cells were treated for 15 minutes with hyaluronic acid fragments (fHAs) (20 μg/ml), interleukin (IL)-1β (5 ng/ml), lipopolysaccharide (LPS) (25 ng/ml) or left untreated and whole cell extracts harvested for Western blot analysis using antibodies specific to phosphorylated and non-phosphorylated SAPK/JNK (54/46) (**A**), ERK 1/2 (44/42) (**B**) and p38 (**C**). (**D, E**) IVD cells were treated with fHA (20 μg/ml) alone (Control) or in combination with MAP kinase inhibitors (10 μM) specific for p38 (SB203580), ERK 1/2 (PD98059) or SAPK/JNK (SP600125) and IL-6 (D) and matrix metalloproteinase (MMP)-3 (E) protein levels determined by specific ELISA (n = 4). In all cases, analyses were carried out in triplicate and values expressed as mean ± S.D. Statistical analysis was performed using Students t-test, * P <0.05, ** P <0.01 as compared to cells treated with fHAs alone (Control).

matrix degrading enzymes, including *MMP-1*, *MMP-3* and *MMP-13*, although significance was not always attained due to the large variations between patient samples. It is envisaged that larger population studies may help to resolve this issue and thus allow for a more accurate assessment of potential fHA-target genes. The induction of such matrix degrading enzymes by fHAs would undoubtedly contribute to IVD catabolism and thus perpetuate the on-going destructive processes within the actively degenerating disc. In addition to increases in mRNA expression levels, we were also able to detect enhanced levels of secreted IL-6 protein following fHA stimulation of IVD cells. This is considered to be of particular importance when assessing the

relevance of fHAs in IVD degeneration *in vivo*, where secreted cytokines are the main protagonists in driving the pain sensation process [3,37].

The pro-inflammatory effects of LMW fHAs on chondrocytes are generally thought to be dependent on their interaction with TLR4 and CD44 [24,25]. In the present report, we utilized both gene silencing and antibody-directed inhibition approaches in an attempt to identify potential signaling pathways responsible for fHA-dependent IVD cell activation. Contrary to expectations, functional loss of either TLR4 or CD44 did not significantly influence fHA-induced IL-6 production in IVD cells. Furthermore, loss-of-function studies involving RHAMM, another potential mediator of fHA-dependent signaling, were also unable to demonstrate any significant decreases in IL-6 secretion by fHA-stimulated IVD cells. However, the stimulatory effects of fHAs were significantly decreased in IVD cells in which TLR2 expression had been effectively suppressed following siRNA treatment. These observations were further corroborated by studies in which TLR2 activity was inhibited through the use of a specific neutralizing antibody. To our knowledge, this is the first report confirming the involvement of TLR2 in fHA-induced cytokine production in human IVD cells.

The NF-κB signal transduction pathway has previously been implicated as a primary means through which fHAs mediate their effects [34,38]. More specifically, NF-κB activation has been reported to mediate the stimulatory effects of fHAs in chondrocytes [22,25]. In addition to NF-κB, signaling pathways involving MAP kinases have also been shown to play a functional role in the transduction of fHA signals [22,34]. In the present report, we were unable to demonstrate NF-κB activation in human IVD cells following treatment with fHAs, although convincing data were obtained which strongly implicated the MAP kinase pathway as being an important regulator of fHA signaling. We could demonstrate strong activation of MAP kinases ERK and JNK in IVD cells following short-term stimulation with fHAs, although p38 appeared to be less responsive to the actions of fHAs. These findings may have important ramifications in terms of identifying possible mechanisms through which fHAs induce both inflammatory and catabolic responses in IVD cells. Certainly, many of the genes up-regulated by fHAs in the current study have previously been confirmed as MAP kinase target genes in IVD cells [39-41]. Indeed, we were able to confirm activation of ERK and JNK but not p38 MAP kinases as being necessary requirements for fHA-mediated IL-6 production in IVD cells. Furthermore, increases in MMP-3 protein production due to fHAs also appeared to be dependent on JNK MAP kinase activity. This may have significant implications when considering therapeutic strategies for treating IVD degeneration and inflammatory pain development, and may, therefore, warrant further investigations into the possible clinical benefits of using MAP kinase inhibition for treating this debilitating disease.

Conclusions

In conclusion, the data provided in the current report provide convincing evidence that fHA-dependent stimulation of human IVD cells is primarily regulated through TLR2-mediated activation of the MAP kinase pathway. Furthermore, our findings offer new insights into the potential molecular mechanisms governing IVD inflammatory pain development in patients with IVD degeneration. Clearly, therefore, further studies are now needed in order to confirm the presence and concentration of fHAs in tissue samples, and thereby allow for an accurate assessment of their true physiological role in IVD degeneration.

Abbreviations

ADAMTS: A Disintegrin And Metalloproteinase with Thrombospondin Motifs; AND: 7-amino-1,3-naphthalenedisulfonic acid; COX: cyclooxygenase; ECM: extracellular matrix; ERK: extracellular signal-regulated kinase; fHAs: hyaluronic acid fragments; HA: hyaluronic acid; IL: interleukin; IVD: intervertebral disc; JNK/SAPK: c-Jun-N-terminal kinase/stress-activated protein kinase; LBP: low back pain; LMW: low molecular weight; LPS: lipopolysaccharide; MAP: mitogen-activate protein; MMP: matrix metalloproteinase; NF-κB: nuclear factor kappa B; RHAMM: receptor for hyaluronan-mediated motility; TBP: TATA-Box binding protein; TLR: toll-like receptor; TNF: tumour necrosis factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LQ participated in all experimental studies and was involved in drafting the manuscript. MK participated in cell isolation and Western blot analyses. AS, MR and JS generated, purified and characterized the HA fragments used in the current study. ANT participated in all siRNA studies. JK participated in the acquisition of IVD cells used in the current study. NB and KW made substantial contributions to the conception and design of the study, and acquisition of funding. MOH made substantial contributions to the conception and design of the study, and interpretation of data. PJR made substantial contributions to the conception and design of the study, interpretation of data, and drafting of the manuscript. All authors read and approved the final manuscript version.

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Curcuma DMSO extracts and curcumin exhibit an anti-inflammatory and anti-catabolic effect on human intervertebral disc cells, possibly by influencing TLR2 expression and JNK activity

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Abstract

Background: As proinflammatory cytokines seem to play a role in discogenic back pain, substances exhibiting anti-inflammatory effects on intervertebral disc cells may be used as minimal-invasive therapeutics for intradiscal/epidural injection. The purpose of this study was to investigate the anti-inflammatory and anti-catabolic potential of curcuma, which has been used in the Indian Ayurvedic medicine to treat multiple ailments for a long time.

Methods: Human disc cells were treated with IL-1β to induce an inflammatory/catabolic cascade. Different extracts of curcuma as well as curcumin (= a component selected based on results with curcuma extracts and HPLC/MS analysis) were tested for their ability to reduce mRNA expression of proinflammatory cytokines and matrix degrading enzymes after 6 hours (real-time RT-PCR), followed by analysis of typical inflammatory signaling mechanisms such as NF-κB (Western Blot, Transcription Factor Assay), MAP kinases (Western Blot) and Toll-like receptors (real-time RT-PCR). Quantitative data was statistically analyzed using a Mann Whitney *U* test with a significance level of $p < 0.05$ (two-tailed).

Results: Results indicate that the curcuma DMSO extract significantly reduced levels of IL-6, MMP1, MMP3 and MMP13. The DMSO-soluble component curcumin, whose occurrence within the DMSO extract was verified by HPLC/MS, reduced levels of IL-1β, IL-6, IL-8, MMP1, MMP3 and MMP13 and both caused an up-regulation of TNF-α. Pathway analysis indicated that curcumin did not show involvement of NF-κB, but down-regulated TLR2 expression and inhibited the MAP kinase JNK while activating p38 and ERK.

Conclusions: Based on its anti-inflammatory and anti-catabolic effects, intradiscal injection of curcumin may be an attractive treatment alternative. However, whether the anti-inflammatory properties *in vitro* lead to analgesia *in vivo* will need to be confirmed in an appropriate animal model.

Keywords: Human intervertebral disc cells, Curcumin, Curcuma, Proinflammatory cytokines, Matrix degrading enzymes, NF-κB, Toll-like receptors, MAP kinase, Back pain, HPLC/MS

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Background

Degeneration of the intervertebral disc is characterized by enhanced proteolytic degradation of extracellular matrix proteins as well as altered matrix protein synthesis. This overall catabolic shift leads to changes in the tissue structure that have been extensively described in the literature [1-7]. Although large structural changes can be observed during degeneration, this age-related process does not necessarily cause pain symptoms.

There is certain evidence in the literature that in a subgroup of patients, painful disc degeneration is characterized by increased levels of proinflammatory cytokines, e.g. interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α) [6,8-11]. Although proinflammatory mediators seem to play a crucial role in intervertebral disc diseases, little is known about inflammatory pathways in intervertebral disc cells. Results from studies on the pathogenesis of cartilage degeneration indicate that proinflammatory processes are mostly regulated by the transcription factor NF-κB [12], whose activity is tightly regulated *in vivo*, e.g. by activation of the so-called Toll-like receptors (TLRs) [13]. Another important inflammatory pathway is the MAP kinase pathway that consists of a family of protein kinases with the major members being p38, ERK and JNK [14,15]. Due to the lack of knowledge concerning the molecular events underlying discogenic back pain, treatment of painful disc disease is currently limited, with typical options for the patient being conservative treatment (e.g. physiotherapy) and oral pain medication, both of which often only have a temporary effect. Other options are various types of surgical interventions, but these lead to high risks for the patients and high costs for the health care systems. Therefore, research in the most recent past has concentrated on the development of minimal-invasive, yet effective new treatment options, covering approaches from cell and gene therapy to anti-inflammatory substances for intradiscal injection. Currently, corticosteroidal substances are frequently used, which are known to have a significant risk for side effects and may cause disc space infections [16]. Although research on biodrugs with regard to spinal diseases is yet rare, these novel anti-inflammatory candidates could potentially benefit patients with discogenic back pain.

Curcuma (*Curcuma longa* L., Zingiberaceae) is a perennial herb that is cultivated in Asian countries. As a powder, it has not only been used for cooking for centuries, but also as a drug in the traditional Chinese and Indian medicine, treating e.g. diabetic wounds, hepatic disorders, rheumatism and sinusitis [17]. Numerous publications demonstrated an anti-inflammatory effect of curcuma, with its effect probably being related to a class of substances called curcuminoids [18].

Based on a thorough literature review, we hypothesize that curcuma has the potential to interfere with catabolic and inflammatory pathways. Hence, the aim of this study was to analyze the effects of curcuma extracts as well as of one selected component of curcuma on IL-1β mediated cellular responses of human intervertebral disc cells *in vitro*. Additionally, its mechanism of action was investigated by testing for involvement of NF-κB, MAP kinases (i.e. p38, ERK, JNK) and TLR2.

Methods

General experimental design

As an inflammatory environment is thought to be present in (a subgroup of) patients with discogenic back pain, human intervertebral disc cells (cultured in 2D) were pretreated with recombinant IL-1β, thus increasing the levels of proinflammatory cytokines and matrix degrading enzymes. Thereafter, different solvents (DMSO, ethanol) were used to prepare sequential curcuma extracts and tested for their ability to reduce inflammatory and catabolic gene expression after 6 hours. The presumably most abundant bioactive substance in the most potent extract was chosen based on structure-based solubility, information in the literature and identification using HPLC/MS analysis (i.e. curcumin) and tested in the same setting, using various concentrations. A mechanistic investigation, looking at involvement of the NF-κB, MAP kinase and TLR2 pathway, was performed for curcumin as well.

Human intervertebral disc cell culture

Human intervertebral disc tissue (nucleus pulposus and annulus fibrosus) was removed from 27 patients undergoing spinal surgery for discectomy or interbody fusion for degenerative disc disease or disc herniation (for detailed information see Table 1). Informed consent was obtained from all patients prior to surgery in accordance with the institutional review board.

Intervertebral disc cells were released from the tissue by enzymatic digestion with 0.2% collagenase NB4 (Serva, Germany) and 0.3% dispase II (Roche Diagnostics, Switzerland) in PBS (37°C, 5% CO_2) for approximately 4 hours. After digestion, the tissue suspension was filtered (70 μm cell strainer, BD Biosciences, Belgium), washed and cells were seeded and expanded in DMEM/F12 (Sigma, Switzerland) supplemented with 10% FCS, penicillin (50 units/ml), streptomycin (50 μg/ml) and ampicillin (125 ng/ml) (all Invitrogen, Germany), with medium changes once to twice a week and expansion up to passage 2 or 3.

Table 1 Demographic data on surgical disc samples (M = male; F = female)

Nr.	Sex	Age	Level	Pathology	Grade	Experiments
1	F	15	L5/S1	Herniation	4	G
2	M	35	L4/5	Herniation	4	G
3	M	46	L3/4	Herniation	3	G
4	M	60	L4/5	Herniation	4	G
5	F	56	L1/2	Herniation	5	G
6	F	48	L4/5	Herniation	4	G
7	M	59	L5/S1	DDD	4	G
8	M	39	L4/5	Herniation	3	G
9	M	60	L4/5	Herniation	3	G
10	F	51	L4/5	DDD	4	G
11	F	48	L4/5	DDD	3	G
12	M	59	L5/S1	Herniation	3	G
13	F	42	L4/5	Herniation	4	G
14	M	28	L5/S1	Herniation	3	V
15	F	71	L1/2	Herniation	5	V
16	F	46	L5/S1	Herniation	5	V
17	M	61	L4/5	Herniation	3	V
18	F	59	L4/5	Herniation	3	V
19	M	57	L4/5	Herniation	4	P
20	M	48	L4/5	Herniation	3	P
21	F	23	L5/S1	Herniation	3	P
22	F	43	L4/5	Herniation	4	P
23	M	49	L5/S1	Herniation	4	P
24	F	50	L5/S1	Herniation	3	P
25	F	45	L5/S1	Herniation	4	P
26	F	40	L4/5	Herniation	4	P
27	N	26	L4/5	Herniation	4	P

Grade = Pfirrmann Grading; Pathology: DDD = Degenerative disc disease
Experiments: V = Viability, G = Gene expression analysis, P = Pathway analysis.

Preparation of curcuma extracts

Organic curcuma from McCormick (Promena, Switzerland) was used to prepare sequential DMSO and ethanol extracts. Briefly, curcuma was dispersed in DMSO at a concentration of 320 mg/ml, incubated on the shaker at room temperature for 10 min and centrifuged at 2000 rpm for 10 min before taking off the DMSO fraction. The remaining pellet was then dispersed in 100% ethanol and the procedure was repeated. After removal of the ethanol fraction, the thereafter remaining pellet was discarded. For each experiment, the fractions were prepared freshly in order to avoid any damage due to freezing/thawing.

HPLC/MS analysis of the curcuma DMSO and EtOH extracts

The DMSO and EtOH extracts of curcuma were analysed by high performance liquid chromatography (1200 Series HPLC, Agilent), coupled to a mass spectrometer (6130 series MS, Agilent). The chromatography of the curcuma extracts was performed according to Wichitnithad *et al.* [19], using a RP-C18 column (Agilent Eclipse Plus, 100 mm × 2.1 mm i.d., 1.8 μm). For identification of the curcuminoids, measurements were carried out with a multimode source (electrospray (ESI) ionization mode: positive mode; drying gas flow: 12 l/min; drying gas temperature: 350°C; nebulizer pressure: 50 psig; fragmentor voltage: 70 V; capillary voltage: 4000 V). The quantification of the most abundant curcuminoids was done at a wavelength of 425 nm, with commercially available curcumin (Sigma Aldrich) as an external standard.

Viability measurement

Cells seeded in 24 well plates were treated with different concentrations of curcuma DMSO extract (25, 50, 100, 250, 500 or 1000 μg/ml), curcuma ethanol extract (25, 50, 100, 250, 500 or 1000 μg/ml) or curcumin (1, 5, 10, 20, 50 or 100 μM - dissolved in DMSO). All experiments were performed in triplicates on cells from 5 independent biopsies. After 6, 18 and 30 hours, toxicity was analyzed using the MTT assay: A fresh sterile solution of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, Switzerland) with a concentration of 0.5 mg/ml in DMEM/F12 was prepared, 500 μl were added to each well and incubated for 4 hours at 37°C. MTT was discarded, cells were lysed with DMSO for 5 min at 37°C and absorbance was measured at 565 nm. Absorbance of treated cells was calculated relative to absorbance of untreated control cells, which was set to 100% (only changes in viability of >10% were considered). Concentrations that were non-toxic even at late time points were chosen for subsequent experiments. Results of the MTT assay were previously shown to be comparable to other viability measurement techniques (DNA content by Picogreen assay; cell counting) [20].

Gene expression analysis

Human intervertebral disc cells were serum starved for 2 hours and then exposed to 5 ng/ml IL-1β (Peprotech, Great Britain) for 2 hours before adding 100 μg/ml curcuma DMSO extract or 100 μg/ml curcuma EtOH extract for 6 hours. Untreated control cells were included to verify the inflammatory and catabolic response induced by IL-1β treatment. As we were able to show that the solvents did not influence cellular behavior (see Additional file 1: Figure S1 and Additional file 2: Figure S2), all groups were treated with the respective volume (0.03%) of either DMSO or EtOH in all experiments. Therefore, changes in gene expression are either calculated relative to controls (+0.03% DMSO or EtOH) or relative to IL-1β prestimulated cells (+0.03% DMSO or EtOH). Based on the results with curcuma extracts

and data obtained by HPLC/MS analysis, a 25 mM stock solution of curcumin (which is one of the major DMSO-soluble, bioactive components of curcuma) was prepared and cells were treated with final concentrations of 5, 10 or 20 μM curcumin for 6 hours after IL-1β prestimulation. Taking the approximate percentage of curcumin in curcuma powder (~2%) into account, the applied range of curcumin (5–20 μM) was predicted to be similar to the final concentration of curcumin when using the above mentioned curcuma extracts (100 μg/ml). All gene expression experiments were performed on cells from five independent biopsies.

After treatment, cells were harvested by trypsin treatment and total RNA was isolated using the PureLink RNA Mini Kit (Ambion, Switzerland) according to the manufacturer's instructions. cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Switzerland) and gene expression of IL-1β, IL-6, IL-8, TNF-α, MMP1, MMP3, MMP13, TLR2 and TBP (TATA box binding protein = housekeeping gene) was analyzed. Human specific probes and primers (Applied Biosystems, Switzerland, see Table 2), TaqMan real-time RT-PCR Mix (Applied Biosystems, Switzerland) and 10–30 ng of cDNA (depending on the expression level of the respective gene) were mixed and measured in duplicates using the StepOne Plus Real-Time PCR System (Applied Biosystems, Switzerland). The comparative ct method (= $2^{-\Delta\Delta Ct}$ method) [21] was used to quantify PCR data. In order to calculate changes in gene expression induced by curcuma/curcumin, gene expression in IL-1β-treated cells was set to 100% and gene expression of IL-1β/curcuma or IL-1β-/curcumin-treated cells was calculated relative to IL-1β-treated cells (containing the respective amount of either DMSO or EtOH as well).

Western Blot for NF-κB (p65)

In order to investigate whether changes in NF-κB/p65 translocation occur after treatment with curcumin (substance with most prominent effects, see results), disc cell cultures were either kept untreated, treated with 5 ng/ml IL-1β alone or co-treated with 20 μM curcumin for 60 min.

Table 2 Primers/Probes used for real-time RT-PCR (TaqMan® Gene Expression Assays, Applied Biosystems)

Gene	Primer sequence number	Base pairs
Interleukin-1β (IL-1β)	Hs00174097_m1	94
Interleukin-6 (IL-6)	Hs00174131_m1	95
Interleukin-8 (IL-8)	Hs00174103_m1	101
Matrixmetalloproteinase-1 (MMP1)	Hs00233958_m1	133
Matrixmetalloproteinase-3 (MMP3)	Hs00968308_m1	98
Matrixmetalloproteinase-13 (MMP13)	Hs00233992_m1	91
TATA box binding protein (TBP)	Hs00427620_m1	91
Toll-like receptor 2 (TLR2)	Hs00152932_m1	80
Tumor necrosis factor alpha (TNF-α)	Hs00174128_m1	80

Data was obtained by real-time RT-PCR (ΔΔct method) (n = 10).

Nuclear extracts were prepared by washing trypsin-harvested cells with 10 mM HEPES (pH 7.9), containing 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 5 mM DTT and 0.1% protease inhibitors (pepstatin-A, leupeptin and bestatin). Then, cells were lysed with 0.1% NP-40 for 5 min, centrifuged for 5 min at 10'000 rpm (4°C) and supernatants were discarded. Nuclear pellets were washed with 0.1% NP-40 and lysed for 20 min with 20 mM HEPES (pH 7.9), containing 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM PMSF and 5 mM DTT as well as protease inhibitors (see above). After centrifugation, protein content was measured by Bradford assay (Bio-rad, Germany).

Nuclear extracts of untreated, IL-1β-treated and IL-1β/curcumin-treated cells were separated on a SDS-polyacrylamid gel and transferred to a PVDF membrane (Amersham, Switzerland). The membrane was incubated with a p65 antibody (Santa Cruz, Germany) followed by incubation with an appropriate HRP secondary antibody before analyzing chemiluminescence. PARP (Poly [ADP-ribose] polymerase) was used as a loading control. The assay was performed on cells from three independent biopsies.

Transcription factor assay for NF-κB (p65)

In order to detect specific NF-κB DNA binding activity in nuclear extracts, the NF-κB (p65) Transcription Factor Assay (Cayman, Estonia) was used according to the protocol provided by the manufacturer. Briefly, a specific double stranded DNA (dsDNA) sequence containing the NF-κB response element was immobilized to the wells of a 96 well plate. Nuclear extracts were prepared as described above and added to the coated wells. NF-κB contained in the added nuclear extract bound specifically to the NF-κB response element and was detected by addition of the provided specific primary antibody directed against NF-κB (p65). A secondary antibody conjugated with HRP was added, a colorimetric readout at 655 nm was performed and data was quantified as indicated in the protocol. The assay was performed on cells from two independent biopsies.

Western blot for MAP kinases (p38, ERK, JNK)

Whole cell extracts of untreated, IL-1β-treated and IL-1β/curcumin-treated cells were prepared after 15 min (using standard protocols) to investigate whether curcumin acts on typical MAP kinases. Protein content was measured by Bradford assay and immunoblotting of whole cell extracts was performed as described for p65, but membranes were incubated with antibodies recognizing

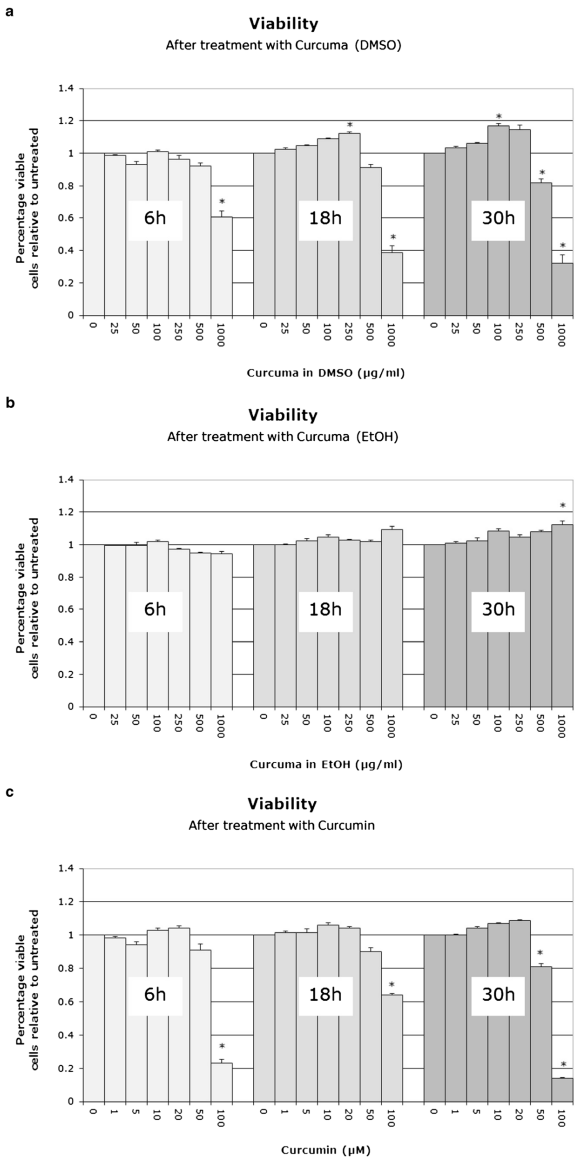

Figure 1 Cytotoxicity of the curcuma DMSO extract (1a), curcuma ethanol extract (1b) and curcumin (1c) after 6, 18 and 30 hours. Data was obtained by use of the MTT assay and is presented as Mean and SEM (n = 5). Asterisks indicate statistical significance ($p < 0.05$).

either unphosphorylated or phosphorylated p38, ERK (p42/44) or JNK (Cell Signaling, USA) before adding an HRP-labeled rabbit secondary antibody and analyzing chemiluminescence. Tubulin was used as a loading control. The assay was performed on samples from five independent experiments.

Statistical analysis

All quantitative data (cytotoxicity, gene expression) was statistically analyzed using a Mann Whitney *U* test on the SPSS statistics software and differences were considered statistically significant at $p < 0.05$ (two-tailed).

Results

Cytotoxicity of curcuma extracts and curcumin

Cytotoxicity of curcuma extracts (DMSO, ethanol) and curcumin was determined after 6, 18 and 30 hours (i.e. toxicity for short and long time points) using the MTT assay. For the curcuma DMSO extract, cell viability was constricted at concentrations of 500 μg/ml (30 hours) and 1000 μg/ml (all time points) (Figure 1a). A slight proliferative effect was observed for 100 μg/ml (30 hours) and 250 μg/ml (18 hours). For the curcuma ethanol extract, no cytotoxic effect could be observed at any time point up to a concentration of 1000 μg/ml (Figure 1b). For curcumin, cytotoxic effects could be observed at concentrations of 50 μM (30 hours) and 100 μM (all time points) (Figure 1c).

Changes in gene expression with IL-1β prestimulation

With IL-1β treatment, we could observe a significant increase in the mRNA levels of all genes of interest at the time of analysis (6 hours). Data for all genes is shown in Table 3 as mean, SEM and p-value (data based on analysis of cells from 10 independent biopsies).

Table 3 Effects of IL-1β stimulation on mRNA levels of candidate genes after 6 hours

Gene	Mean (Fold Change)	SEM	p-Value
IL-1β	246.34	48.12	<0.001
IL-6	2895.17	1571.28	<0.001
IL-8	241.89	56.71	<0.001
MMP1	166.05	31.06	<0.001
MMP3	260.58	70.75	<0.001
MMP13	119.06	29.49	<0.001
TNF-α	21.45	7.16	<0.001
TLR2	8.49	1.64	<0.001

Data was obtained by real-time RT-PCR (ΔΔct method) (n = 10).

Changes in gene expression with curcuma DMSO and ethanol extracts

As shown in the Supplementary Material (Additional file 1: Figure S1 and Additional file 2: Figure S2), neither DMSO nor EtOH at the used concentration (0.03%) influenced the expression of the inflammatory and catabolic target genes.

Treatment with the curcuma DMSO extract resulted in a significant inhibition of MMP1, MMP3 and MMP13 after 6 hours, relative to IL-1β prestimulated cells (which are also supplemented with the respective volume of DMSO). While no changes occurred in the expression of IL-1β and IL-8, a significant inhibition of IL-6 was observed. However, we noticed a strong induction of TNF-α expression at this early time point. Expression of TLR2 was significantly reduced. For all results see Figure 2a as well as Additional file 3: Table S3 for summarized values.

Compared to IL-1β prestimulated cells, treatment with the curcuma EtOH extract did not cause any changes in gene expression after 6 hours for MMP1 and MMP3 while slightly decreasing MMP13 expression. Expression of IL-1β, IL-6 and IL-8 also remained unchanged, but TNF-α expression was increased. TLR2 expression was not influenced. For all results see Figure 2b as well as Additional file 3: Table S3 for summarized values.

Analysis of the curcuma DMSO and EtOH extracts (HPLC/MS)

Based on the above shown results, the DMSO fraction seemed to contain one or more anti-catabolic and anti-inflammatory substances. Taking the solubility of the various components of curcuma as well as the literature-based preselection of anti-inflammatory components of curcuma into account, the curcuminoid curcumin was chosen to be the most promising candidate substance with biological activity. In order to proof that curcumin was indeed present in the DMSO extract, HPLC/MS analysis was performed on the stock extracts (320 mg/ml). The results showed that predominantly curcumin (6.32 mg/ml) (Peak 1) was present in the extract (retention time 16.9 min, $(M + H)^+$ at m/z 369.1), followed by its precursors demethoxycurcumin (retention time 15.4 min, $(M + H)^+$ at m/z 339.1) (Peak 2) and bisdemethoxycurcumin (retention time 13.7 min, $(M + H)^+$ at m/z 309.1) (Peak 3) and other unidentified compounds with little absorbance (Figure 3).

As curcumin is also soluble in EtOH, we performed a sequential extraction process described under Materials and Methods in order to aggregate curcumin in the DMSO extract. Both, the sequential EtOH extract (stock extract = 320 mg/ml) as well as the pure curcumin stock solution in DMSO (25 mM) were also measured by HPLC/MS. While the curcuma DMSO extract contained 6.32 mg/ml of curcumin, the sequential

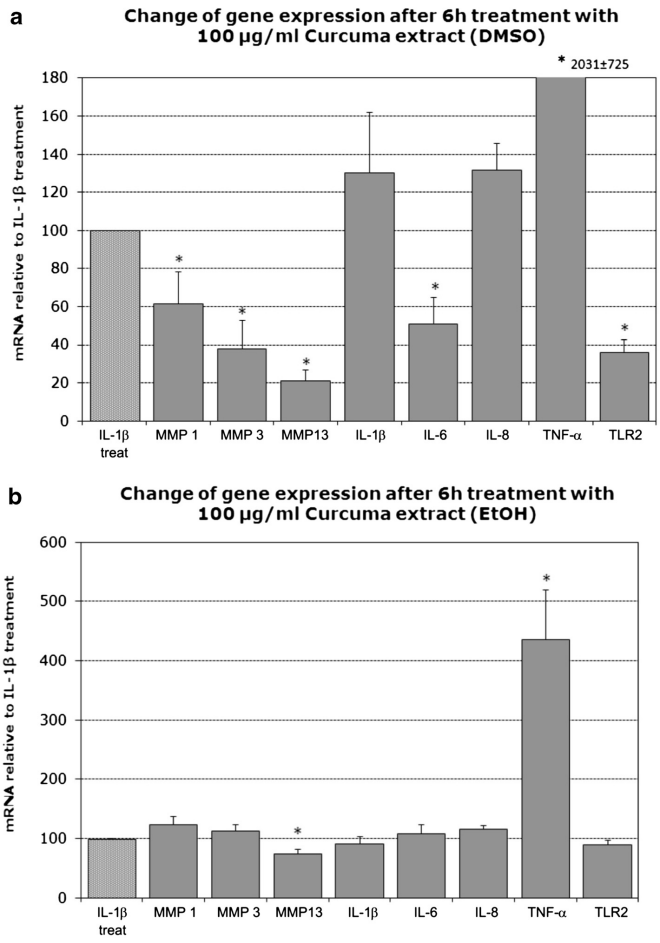

Figure 2 Effects of the curcuma DMSO extract (2a) and curcuma ethanol extract (2b) on mRNA levels of candidate genes after 6 hours, indicated as fold change relative to IL-1β-treatment (IL-1β treated cells also contain 0.03% of DMSO or EtOH respectively). Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta C_T}$ method) and is presented as Mean and SEM (n = 5). Asterisks indicate statistical significance ($p < 0.05$). Each gene was normalized to its respective IL-1β treatment (IL-1β + solvent), which was always set to 100% (only one exemplary control bar is shown).

curcuma EtOH extract contained only 1.2 mg/ml (Figure 4). Curcumin itself showed the highest value and was in a similar range as the curcuma DMSO extract (8.80 mg/ml) (Figure 4).

Changes in gene expression with curcumin

Based on the above-mentioned findings, curcumin was investigated at different concentrations (equalizing to the approximate concentration of curcumin in the curcuma extract) in more detail at the 6 hour time point. Treatment with curcumin caused a significant reduction of MMP1 (Figure 5a) and MMP3 (Figure 5b) at 10 μM and 20 μM. For MMP13, all concentrations of curcumin caused a significant reduction (Figure 5c). Expression of IL-1β (Figure 5d) and IL-6 (Figure 5e) was significantly inhibited at both, 10 μM and 20 μM, while the lowest

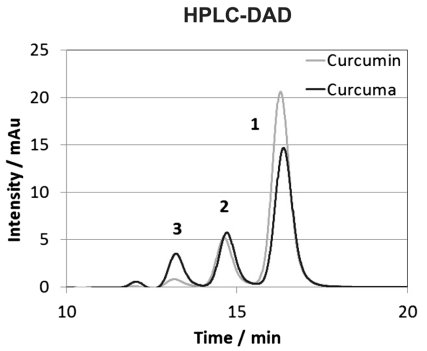

Figure 3 Analysis of the composition of the curcuma DMSO extract (black line) and the reference substance curcumin (grey line, >94% purity with demethoxycurcumin and bisdemethoxycurcumin as impurity) in DMSO by HPLC-DAD (detection wavelength 425 nm). The three peaks were identified as curcumin (1, retention time 16.9 min), demethoxycurcumin (2, retention time 15.4 min) and bisdemethoxycurcumin (3, retention time 13.7 min) by their $(M+H)^+$ ions in the HPLC/MS analysis.

concentration caused a slight increase of IL-6. IL-8 expression was also decreased at 20 μM (Figure 5f). In contrast, TNF-α expression was significantly increased at all three curcumin concentrations, with the most prominent effects at 20 μM (Figure 5g). Furthermore, TNF-α expression was also increased upon curcumin treatment alone (i.e. if no IL-1β pretreatment was performed), while all other target genes remained unaltered under these conditions (data not shown). TLR2 expression was significantly reduced with each concentration (Figure 5h). For summarized values see Additional file 4: Table S4.

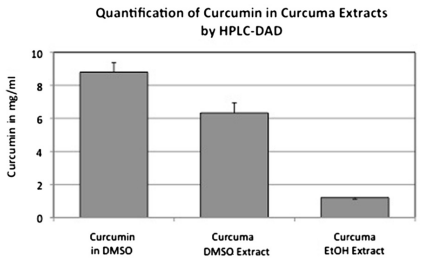

Figure 4 HPLC/MS measurement of the curcumin concentration in curcuma DMSO and curcuma EtOH stock extracts (320 mg/ml), prepared by the described sequential extraction procedure. In addition, the curcumin stock solution (25 mM; in DMSO) was quantified.

Analysis of NF-κB

Immunoblotting of p65 in nuclear extracts of untreated, IL-1β-treated and IL-1β/curcumin-treated cells revealed that IL-1β-treatment caused nuclear translocation of p65 after 60 min. However, compared to IL-1β stimulated samples, curcumin treatment did not reduce levels of the target protein in nuclear extracts (equal protein loading was confirmed by PARP detection) (Figure 6a). Using the NF-κB/p65 transcription factor assay, we provide further evidence that IL-1β strongly induced NF-κB DNA binding (similar to the positive control = Pos Ctrl), while curcumin was not able to reduce levels after IL-1β stimulation (Figure 6b). Internal assay controls (i.e. competition and non-specific binding control) ensured validity of the test.

Analysis of MAP kinases (p38, ERK, JNK)

Effects of curcumin on MAP kinase activity were investigated by detection of levels of phosphorylated and unphosporylated p38 (Figure 7a), ERK (Figure 7b) and JNK (Figure 7c) using immunoblotting technique of whole cell extracts. Results demonstrate that IL-1β treatment increased levels of phosporylated p38, ERK and JNK after 15 min, which is indicative of activation of these MAP kinases. Treatment with curcumin reduced activity of JNK compared to IL-1β treatment (i.e. reduced levels of p-JNK), but further increased levels of p-ERK and p-p38 compared to IL-1β treatment. Levels of unphosporylated p38, ERK and JNK were similar in all groups. Equal protein loading was confirmed by tubulin detection.

Discussion

Changes in gene expression

Curcuma is not only an ancient spice, but also a traditional remedy that has been used in Indian and Chinese medicine to treat indigestion and many other medical issues. Since the 1970s, the anti-inflammatory compounds called curcuminoids were discovered in the spice, with one (and probably the most important component) being curucmin [22]. Because of its anti-inflammatory properties, curcuma and its components (especially curcumin) have been investigated in osteoarthritis and rheumatoid arthritis during the past one to two decades, while only one paper has been published on the effects of curcumin on intervertebral disc cells so far [23].

Our results clearly show that the different curcuma extracts influenced cellular behavior in a different manner. While the curcuma EtOH extract (which contained only little amounts of curcumin, as shown by HPLC/MS) had no effect and was thus considered to be not suitable for further investigations, the curcuma DMSO extract as well as the DMSO-soluble compound curcumin (which was shown to be present in the DMSO extract by

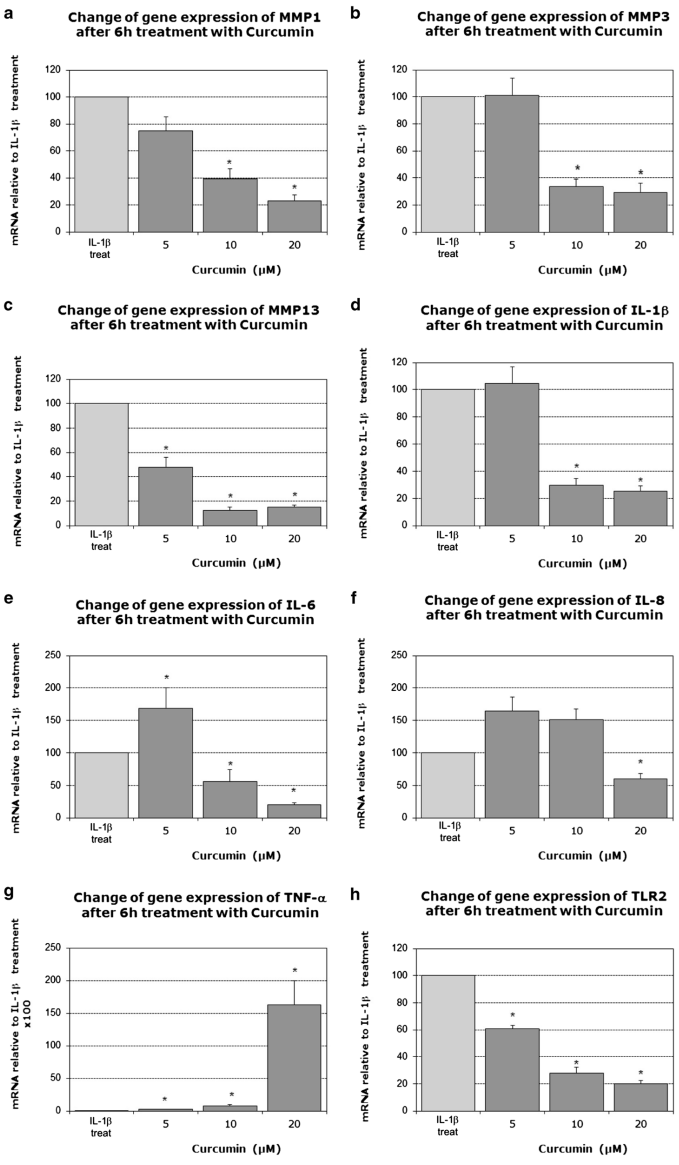

Figure 5 (See legend on next page.)

(See figure on previous page.)

Figure 5 Effects of curcumin (5, 10 or 20 μM) on mRNA levels of candidate genes after 6 hours, indicated as fold change relative to IL-1β-treatment: MMP1 (5a); MMP3 (5b); MMP13 (5c); Interleukin-1β (5d); Interleukin-6 (5e); Interleukin-8 (5f); TNF-α (5g); TLR2 (5h) (IL-1β treated cells also contain 0.03% of DMSO). Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 5). Asterisks indicate statistical significance ($p < 0.05$). Each gene was normalized to its respective IL-1β treatment (IL-1β + DMSO), which was always set to 100% (only one exemplary control bar is shown).

HPLC/MS) reduced levels of some disc-specific, major proinflammatory cytokines and matrix degrading enzymes in our *in vitro* experiments. We were able to demonstrate that the observed effect was not due to the biologic activity of the solvents DMSO and EtOH (Additional file 1: Figure S1 and Additional file 2: Figure S2), although the anti-inflammatory properties of DMSO have most recently been described in human intestinal cells [24].

Specifically, we could demonstrate a reduction in gene expression of IL-6, MMP1, MMP3 and MMP13 when treating IL-1β prestimulated cells with the curcuma DMSO extract. Additionally, IL-1β and IL-8 were reduced by curcumin treatment after 6 hours. As effects were comparable between the curcuma DMSO extract and curcumin and as curcumin was detected at high concentrations in the DMSO extract by HPLC/MS, we hypothesize that the major bioactive substance in curcuma DMSO extracts acting on human intervertebral disc cells could be curcumin. Due to the beneficial effects of curcumin, this natural compound may be of benefit for patients with inflammation-related back pain, with the potential mode of application being intradiscal injection. Albeit curcumin is

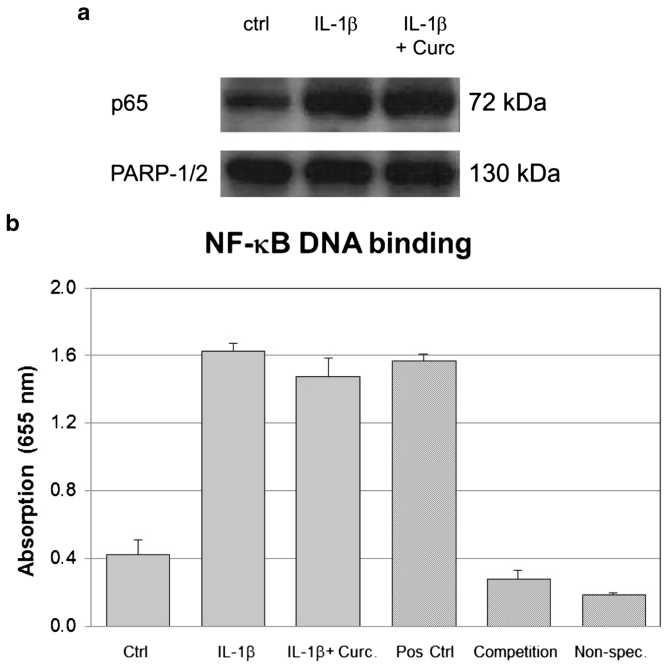

Figure 6 Effects of curcumin on nuclear translocation of p65/NF-κB, measured by immunoblotting of p65 (n = 3, nuclear extracts, 60 min). 6a: Nuclear translocation of p65, measured by immunoblotting (n=3, nuclear extracts, 60 min). One representative sample is shown and PARP-1/2 is used as a loading control.
6b: NF-kB DNA binding activity, measured by NF-kB transcription factor assay (n=2, nuclear extracts, 60 min: without statistical evaluation). Note that different internal controls (positive control, competition control and unspecific binding control) were used to validate the test.

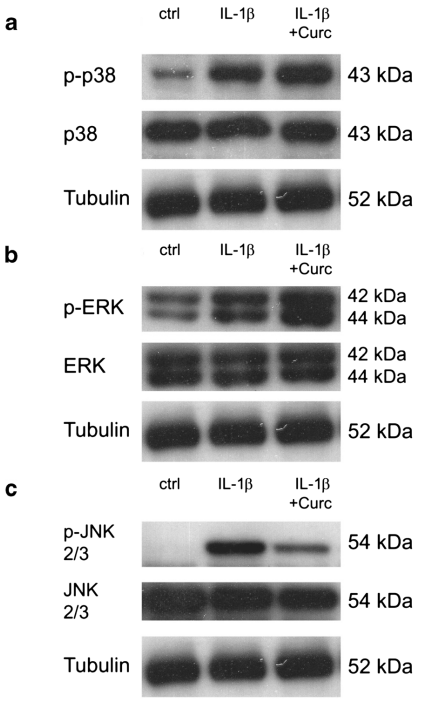

Figure 7 Effects of curcumin on MAP kinase activity, measured by immunoblotting of p38/p-p38 (7a), ERK/p-ERK (7b) and JNK/p-JNK (7c) (n = 5, whole cell extracts, 15 min). One representative sample is shown and tubulin is used as a loading control.

well known for its low bioavailability in case of oral consumption, *in vivo* concentrations after injections should not be a limiting factor.

The observed gene expression results are similar to effects that were observed when treating other cell types with curcumin, e.g. leading to a reduction in IL-1β [25-28], IL-6 [25,28-30], IL-8 [25,31], MMP1 [32], MMP3 [26,32,33] and MMP13 [32,34]. For IL-6, we observed a slight increase at the lowest concentrations (1.5 fold), but a decrease at higher concentrations. This may be due to biphasic effects of curcumin that are based on its dual function to either scavenge or produce reactive oxygen species [35]. However, the biphasic nature of curcumin cannot explain that higher concentrations of curcumin strongly stimulated expression of TNF-α in human intervertebral disc cells (both, without pretreatment as well as after IL-1β prestimulation), which is different from what is described in the literature [25,28,36]. Based on the current study we do not know whether this effect would also occur on the protein level and *in vivo*. Therefore, further studies are thus required to demonstrate safety and usefulness of curcumin in human application.

So far, only one study investigated the effect of curcumin on human intervertebral disc cells [23]. This study tested curcumin for its effects on matrix protein gene expression, but not on the expression of proinflammatory cytokines or matrix degrading enzymes. Results of Yu *et al.*'s study indicated that curcumin is also able to attenuate an IL-1 induced inhibition of SOX9 and collagen–II expression at 20 μg/ml (= 54.3 μM), which is higher than the concentrations used in the present study and which was shown to be a damaging concentration for other (disc-related) cell types (e.g. C-28/I2 = a chondrocyte cell line) [37]. Furthermore, it has to be noted that both, Yu's as well as our study were performed in a 2D culture system, which can cause certain phenotypic changes of disc cells and may thus possibly influence cellular behavior to the tested treatment.

Pathway analysis

Curcumin seems to exhibit its anti-inflammatory and anti-catabolic effects through versatile mechanisms. So far, in different cell types, mainly inhibition of NF-κB, MAP kinases and Toll-like receptors have been shown to play a role [31,38-41].

NF-κB

Inhibition of the transcription factor NF-κB is the best described mechanism of action of curcumin in the literature [42]. A recent study in chondrocytes showed that curcumin inhibits phosphorylation and degradation of IκBα (= nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and thus translocation of the p65 subunit of NF-κB to the nucleus, indicating that inhibition of the NF-κB pathway takes place at a step before IκBα phosphorylation [41]. In intestinal epithelial cells, curcumin seems to exert its effects by blocking a signal leading to IKK (= IκB kinase) activity [31]. However, in our experimental setting, curcumin did not seem to reduce IL-1β induced nuclear translocation of NF-κB/p65 or NF-κB DNA binding, which is in contrast to data obtained by Yu *et al*. on intervertebral disc cells [23].

Toll-like receptors

We were able to demonstrate a down-regulation of TLR2 mRNA expression after treating IL-1β prestimulated IVD cells with curcumin, which confirms findings in other cell types such as monocytic THP-1 cells, HL-60 promyelocytic leukemia cells and primary peripheral blood polymorphonuclear neutrophils [38]. However, in a leukemia cell line, Reuter *et al*. showed an increase in

TLR2 due to curcumin, although most inflammatory mediators were simultaneously down-regulated in this study [25]. There is also some evidence in the literature that curcumin can reduce expression levels of TLR4 [29,43]. Based on how little is known about TLRs and curcumin so far, more research is needed to establish a causal relationship between therapeutic efficacy of curcumin and TLR2 activity.

MAP kinases

The mitogen-activated protein (MAP) kinase signaling pathways, including JNK, p38 and extracellular-signal regulated kinase (ERK), play an important role in the regulation of inflammatory responses [14]. As MAP kinases are regulated by phosphorylation cascades, their activity can be determined by detecting phosphorylation levels. We found that curcumin was able to inhibit phosphorylation of JNK in IL-1β prestimulated IVD cells, which is similar to primary chondrocytes [34,44]. Importantly, pharmacological inhibition of JNK (by SP600125) has previously been shown to suppress MMP1, MMP3 and MMP13 mRNA expression in bovine and murine IVD cells [45,46] (reviewed in [47]).

In contrast, phosphorylation of p38 and ERK was induced upon curcumin treatment in IL-1β prestimulated IVD cells as well as in curcumin-only treated IVD cells, with a synergistic effect of IL-1β and curcumin [48-50]. It may be possible that activation of p38 and ERK led to the up-regulation of TNF-α expression which was observed when IL-1β pretreated and untreated IVD cells were exposed to curcumin, but our experimental design does not allow to establish a causal relationship between MAP kinase activation and TNF-α expression. Furthermore, activation of ERK and p38 does not only control inflammation, but also several other cellular functions, such as cell cycle progression for ERK (i.e. transition from the G1 to the S phase of the cell cycle) [51] and cell growth and differentiation for p38 [52], indicating that MAP kinase related cellular control is of high complexity.

Conclusion

In conclusion, the results of this study demonstrate that curcumin may become an attractive alternative for the treatment of discogenic back pain when envisaging an intradiscal injection method, which will circumvent the low bioavailability of curcumin. Although we were able to show in a previous study (using a similar experimental set-up) that another anti-inflammatory substance (resveratrol), caused pain-reducing effects in a rodent model of radiculopathic pain *in vivo* [53], the analgetic effect of curcumin first needs to be confirmed before clinical trials are reasonable.

Additional files

Additional file 1: Figure S1. Effects of 0.03% DMSO on mRNA levels of candidate genes after 6 hours, indicated as fold change relative to DMSO-free (i.e. untreated) controls (set to 1). Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 3). Each gene was normalized to its respective DMSO-free control, which was always set to 1 (only one exemplary untreated control bar is shown).

Additional file 2: Figure S2. Effects of 0.03% EtOH on mRNA levels of candidate genes after 6 hours, indicated as fold change relative to EtOH-free (i.e. untreated) controls (set to 1). Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 3). Each gene was normalized to its respective EtOH-free control, which was always set to 1 (only one exemplary untreated control bar is shown).

Additional file 3: Table S3. Summarized values of the graphical illustration of the effects of the curcuma DMSO and EtOH extracts shown in Figure 1**.** Quantitative values of the anti-catabolic and anti-inflammatory effects of the curcuma DMSO and EtOH extracts on mRNA levels of candidate genes after 6 hours (indicated as fold change relative to IL-1β-prestimulation: 100%) are given only if a ***statistically significant reduction*** occurred ($p < 0.05$). Note that IL-1β prestimulated cells also contain 0.03% of DMSO or EtOH respectively. Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 5).

Additional file 4: Table S4. Summarized values of the graphical illustration of the effects of curcumin shown in Figure 5. Quantitative values of the anti-catabolic and anti-inflammatory effects of curcumin on mRNA levels of candidate genes after 6 hours (indicated as fold change relative to IL-1β-prestimulation: 100%) are given only if a ***statistically significant reduction*** occurred ($p < 0.05$). Note that IL-1β prestimulated cells also contain 0.03% of DMSO. Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 5).

Competing interests

All authors declare that they have no competing interests.

Authors' contributions

MK carried out the cell culture experiments, performed statistical analysis and helped to draft the manuscript. LQ carried out the cell culture experiments, performed statistical analysis and helped to draft the manuscript. JK participated in the design of the study, provided clinical sample and medical scientific input and helped to draft the manuscript. AG designed and carried out the HPLC/MS experiments together with BK and helped to draft the manuscript. BK designed and carried out the HPLC/MS experiments together with AG and helped to draft the manuscript. OH participated in the design of the study, provided clinical sample and medical scientific input and helped to draft the manuscript. NB participated in the design of the study, conceived funding for the study and helped to draft the manuscript. KW conceived funding of the study, designed and coordinated the study, performed statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

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Human MMP28 expression is unresponsive to inflammatory stimuli and does not correlate to the grade of intervertebral disc degeneration

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Abstract

Background: MMP28 (epilysin) is a recently discovered member of the MMP (matrix metalloproteinase) family that is, amongst others, expressed in osteoarthritic cartilage and intervertebral disc (IVD) tissue. In this study the hypothesis that increased expression of MMP28 correlates with higher grades of degeneration and is stimulated by the presence of proinflammatory molecules was tested. Gene expression levels of MMP28 were investigated in traumatic and degenerative human IVD tissue and correlated to the type of disease and the degree of degeneration (Thompson grade). Quantification of MMP28 gene expression in human IVD tissue or in isolated cells after stimulation with the inflammatory mediators lipopolysaccharide (LPS), interleukin (IL)-1β, tumor necrosis factor (TNF)-α or the histondeacetylase inhibitor trichostatin A was performed by real-time RT PCR.

Results: While MMP28 expression was increased in individual cases with trauma or disc degeneration, there was no significant correlation between the grade of disease and MMP28 expression. Stimulation with LPS, IL-1β, TNF-α or trichostatin A did not alter MMP28 gene expression at any investigated time point or any concentration.

Conclusions: Our results demonstrate that gene expression of MMP28 in the IVD is not regulated by inflammatory mechanisms, is donor-dependent and cannot be positively or negatively linked to the grade of degeneration and only weakly to the occurrence of trauma. New hypotheses and future studies are needed to find the role of MMP28 in the intervertebral disc.

Keywords: MMP28, Epilysin, Matrix metalloproteinase, Intervertebral disc, Inflammation

Background

Proteins of the matrix metalloproteinase (MMP) family play an essential role in tissue homeostasis by initiating breakdown and reorganization of the extracellular matrix. While being tightly regulated in normal physiological processes (e.g. via tissue inhibitors of metalloproteases TIMPs), dysregulation of MMPs has been implicated in many diseases. During intervertebral disc (IVD) degeneration, the expression and activity of a number of MMPs is increased, including MMPs 1, 3, 7, 9 and 13 [1]. Proinflammatory cytokines such as IL-1β and TNF-α as well as bacterial endotoxins (e.g. lipopolysaccharide LPS) can stimulate expression of various MMPs (e.g. MMPs 1, 3, 9 and 13) in the IVD, as well as in cartilage [2-10].

During the recent past, five new members in the MMP family have been identified: MMP24 to MMP28. MMP28, also known as epilysin and most closely related to MMP19, is a soluble MMP that contains an activation sequence recognized by the furin endoprotease following the pro-domain [11]. It is a well-conserved MMP, with great similarity (97%) in the catalytic domain between human and mouse and overall 85% identical amino acids [12]. MMP28 is strongly expressed in testis, as well as in bone, kidneys, lung, heart, colon, intestines, brain, skin and carcinomas [12-17]. It is also expressed in cartilage, synovium and IVDs, with lower expression in bovine discs compared to bovine cartilage [18-22]. Interestingly, MMP28 expression seemed to be

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increased in osteoarthritis and degenerated IVD compared to healthy tissue, indicating that it may play an important role during these disease processes [18-21].

Despite increasing interest in the role of MMP28 in vivo, little is known about its substrates. Recombinant MMP28 has been reported to degrade casein in vitro and is thought to cleave several neural proteins such as neurite outgrowth inhibitor A (= Nogo-A), neural cell adhesion molecule (= NCAM-1) and neuregulin 1 (= NRG1) [17,23,24]. However, with regard to musculoskeletally relevant proteins, no information on potential substrates is currently available.

As symptomatic degenerated IVDs are characterized by increased levels of certain proinflammatory mediators [10,25-27], which are know to regulate several MMPs [1], we hypothesized that MMP28 expression could be increased in an inflammatory context. Therefore, the aim of this study was to determine the expression level of MMP28 in traumatic or degenerated discs (with different degrees of degeneration) and to investigate the effects of different concentrations of the proinflammatory mediators IL-1β, TNF-α or LPS on its expression in human IVD cells at various time points. Additionally, the effect of the histondeacetylase (HDAC) inhibitor trichostatin A was investigated, as it has been shown to be an up-regulator of MMP28 expression in HeLa cells [28].

Materials and methods

MMP28 expression in human IVD biopsies

Thirteen tissue samples from eight patients who had been diagnosed with symptomatic degenerative disc disease or spinal trauma were included in this part of the study. Based on magnetic resonance imaging (MRI) findings, the degree of IVD degeneration was evaluated according to the Thompson grading system prior to the surgical intervention (for detailed information see Table 1) [29]. Informed consent was obtained from all patients according to the local ethical regulations. Frozen biopsies were pulverized, the IVD fragment powder was dissolved in 1 ml of TriFast RNA isolation reagent (PeqLAB) and total RNA was isolated according to the manufacturer's instructions. cDNA was prepared from total RNA using VILO cDNA Synthesis Kit (Invitrogen). For Real-Time (RT)-PCR, cDNA template (5 μl) was mixed with the qPCR reaction solution (IQ SYBR Green Supermix, Bio Rad) and expression of GAPDH and MMP28 was measured:

GAPDH: Forward-TGGACTCCACGACGTACTCA
GAPDH: Reverse-GGAAGCTTGTCATCAATGGAA
MMP28: Forward-GCCGTGCAGAGCCTGTAT
MMP28: Reverse-GAGTCCCAGGTCTCAAAGTCA
Furthermore, MMP13 was measured as a control gene:
MMP13: Forward-CCAGTCTCCGAGGAGAAACA
MMP13: Reverse-AAAAACAGCTCCGCATCAAC

Table 1 Clinical and demographic data of the study population used for ex vivo gene expression analysis (age, sex, diagnosis, Thompson grade [29], vertebral level)

Sample	Sex	Age	Diagnosis	Grade	Level	Region
1	M	31	DDD	II	L5/S1	NP
2a	F	39	Trauma	II	C4/5	AF
2b	F	39	Trauma	II	C4/5	NP
3a	F	57	Trauma	II	Th10/11	AF
3b	F	57	Trauma	II	Th10/11	NP
4a	F	70	Trauma	II	Th12/L1	AF
4b	F	70	Trauma	II	Th12/L1	NP
5a	M	29	Trauma	III	L1/2	AF
5b	M	29	Trauma	III	L1/2	NP
6a	F	34	DDD	III	L4/5	AF
6b	F	34	DDD	III	L4/5	NP
7a	M	41	DDD	III	C4/5	AF
7b	M	41	DDD	III	C4/5	NP
8	M	46	DDD	III	L5/6	NP
9a	M	25	DDD	IV	L5/S1	AF
9b	M	25	DDD	IV	L5/S1	NP
10	M	44	DDD	IV	L5/S1	AF+NP
11	M	50	DDD	IV	L5/S1	AF+NP
12	M	55	Spondylodesis	IV	C3/4	AF+NP
13a	F	58	Spondylodesis	IV	C5/6	AF
13b	F	58	Spondylodesis	IV	C5/6	NP
14	M	37	DDD	V	L5/S1	AF+NP
15a	F	41	DDD	V	L3/4	AF
15b	F	41	DDD	V	L3/4	NP
16a	M	67	DDD	V	L5/S1	AF
16b	M	67	DDD	V	L5/S1	NP
17a	M	72	DDD	V	L4/5	AF
17b	M	72	DDD	V	L4/5	NP

M = male, F = female, NP = nucleus pulposus, AF = annulus fibrosus. DDD = degenerative disc disease, C = cervical, Th = thoracic, L = lumbar, S = sacral (degeneration grade according to Thompson)

Primers were used at a concentration of 0.25 nM, reactions were carried out in triplicates and the specificity of the amplification products was controlled with a melting curve analysis of each reaction. The $2^{-\Delta Ct}$ method was used to calculate gene expression levels of MMP28 and MMP13. To assure consistent PCR quality, a functional cDNA quality control was used. Samples that produced Ct values for GAPDH greater than 26 were not included in the analysis. Instead PCR was repeated with a new sample with identical Thompson grade.

Isolation, culture and stimulation of IVD cells

Twenty patients who had been diagnosed with symptomatic disc disease or disc herniation (for detailed

information see Table 2) and had undergone operative treatment were included in this cell culture study. Informed consent was obtained from all patients according to the local ethical regulations. Disc tissue was minced and treated with 0.3% collagenase NB4 (Serva) and 0.2% dispase II (Roche Diagnostics) in phosphate buffered saline (PBS) for approximately 6 hours at 37°C. After digestion, the cell suspension was filtered using a 70 μm cell strainer (BD Bioscience, Switzerland), centrifuged at 1000 g for 5 min and the cell pellet was washed with and then resuspended in DMEM/F12 (Sigma). Cells were expanded in a 2D culture containing DMEM/F12 (Sigma) with 10% FCS (Tecomedical), penicillin (50 U/mL), streptomycin (50 μg/mL), and ampicillin (125 ng/mL) (Gibco), with medium changes twice a week. When an 80% confluence level was reached, expanded cells in passage 2 or 3 were rendered serum free for 2 hours and, in a first set of experiments, incubated with LPS, IL-1β and TNF-α in a time-dependent (n = 5) and dose-dependent manner (n = 5). For the dose dependency experiment, cells were treated for 18 hours with different concentrations of LPS (0.01 μg/ml, 0.1 μg/ml, 1.0 μg/ml, 2.0 μg/ml), IL-1β (0.1 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml) or TNF-α (0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml). For the time course experiment, cells were incubated with one chosen concentration of LPS (1 μg/ml), IL-1β (5 ng/ml) or TNF-α (100 ng/ml) for 2, 6 or 18 hours in serum-free medium. In a second set of experiments, disc cells as well as HeLa cells (positive control) were incubated with different concentrations of the HDAC inhibitor trichostatin A (1 nM, 10 nM, 100 nM, 1000 nM) (Sigma-Aldrich) for 18 hours (n = 3). As trichostatin A is dissolved in EtOH, a respective EtOH control was included in these experiments. All concentrations of all chemicals were shown to be non-toxic in advance using the MTT assay (data not shown).

Table 2 Clinical and demographic data of the study population used for in vitro cell culture experiments (age, sex, diagnosis, Pfirrmann grade [38], vertebral level)

Sample	Age	Sex	Diagnosis	Grade	Level
1	59	F	Disc Herniation	4	L4/5
2	61	M	Disc Herniation	4	L3/4
3	50	M	Disc Herniation	3	L5/S1
4	46	F	Disc Herniation	5	L5/S1
5	51	M	Symptomatic Disc Disease	3	L4/5
6	50	F	Disc Herniation	3	L3/4
7	51	F	Disc Herniation	3	L4/5
8	47	M	Disc Herniation	5	L4/5
9	42	M	Disc Herniation	4	L4/5
10	80	M	Disc Herniation	4	L2/3
11	50	M	Disc Herniation	4	L4/5
12	48	F	Symptomatic Disc Disease	3	L4/5
13	61	F	Disc Herniation	4	L4/5
14	37	F	Disc Herniation	4	L4/5
15	66	M	Disc Herniation	3	L5/S1
16	70	F	Disc Herniation	4	L5/S1
17	40	F	Disc Herniation	4	L4/5
18	55	F	Disc Herniation	3	L4/5
19	26	M	Disc Herniation	4	L5/S1
20	57	M	Disc Herniation	4	L4/5

M = male, F = female, L = lumbar, S = sacral (grading according to Pfirrmann)

MMP28 mRNA detection in isolated human IVD cells after stimulation

After stimulation, cells were trypsinized and total RNA was isolated according to the manufacturer's recommendation (PureLink RNA Mini Kit, Invitrogen). For each sample, 1 μg of total RNA was reverse transcribed to cDNA (Reverse Transcription Reagents, Applied Biosystems) and then used for real-time RT-PCR measurements using TaqMan Gene Expression assays (Applied Biosystems) for detection of MMP28 (Hs00425233_m1) as well as of TATA-box binding protein TBP (internal control) (Hs00427620_m1). As a positive control, expression of MMP13 was also measured (Hs00233992_m1) on samples stimulated with IL-1β (10 ng/ml), LPS (2.0 μg/ml) or TNF-α (100 ng/ml) for 18 hours.

Gene expression was first normalized to the housekeeping gene before comparing expression of treated cells to untreated control or the respective solvents control if applicable ($2^{-\Delta\Delta Ct}$ method). Only changes > 2-fold were considered to be relevant.

Statistical analysis

To compare gene expression levels between the study groups, the Wilcoxon signed-rank test was used to determine significance between the groups. The statistical software package SPSS was used and the significance level was set to $p < 0.05$

Results

MMP28 gene expression pattern in human disc tissue

Analysis of MMP28 gene expression in disc biopsies, which was grouped according to the degree of IVD degeneration (Thompson grade), is shown in Figure 1a: MMP28 was expressed in most of the analyzed disc samples and higher expression levels were found in samples removed because of spine trauma (Thomson grade II = normal adult discs with no disc degeneration). Expression levels were low or practically absent in samples with Thompson grade III (i.e. mild disc degeneration), but increased slightly with increasing disc degeneration, with high donor-donor variation. No consistent statistically

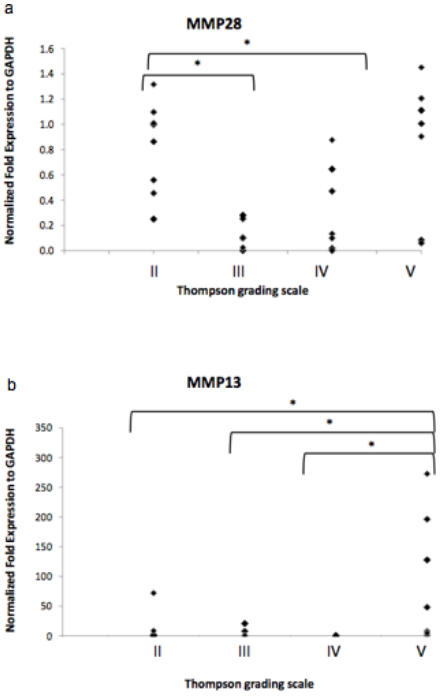

Figure 1 Ex vivo human IVD tissue gene expression of a) MMP28 and b) MMP13 (= positive control) in degenerated or traumatic samples with Thompson grading II-V (patients: n = 17, samples: n = 28). Data is presented as individual measurement values; * if $p < 0.05$ between indicated groups.

significant correlation between MMP28 expression and Thompson grades or disease could be found (Figure 1a). As a control gene, MMP13 expression was analyzed in the same samples, and it showed a strong increase in expression in samples with Thompson grade V degeneration (Figure 1b), as previously described in the literature [30,31].

Regulation of MMP28 gene expression

No changes in MMP28 expression could be observed when cells were treated with different concentrations of LPS (Figure 2a), IL-1β (Figure 2b) or TNF-α (Figure 2c) for 18 hours, no matter which concentration was used. As changes in gene expression may strongly depend on the chosen time point, one concentration that is typically used in the literature was chosen for each inflammatory mediator and cellular behavior was investigated after 2, 6 or 18 hours of treatment. However, even at different time points, MMP28 expression was not regulated by LPS (1 μg/ml) (Figure 3a), IL-1β (5 ng/ml) (Figure 3b) or TNF-α (100 ng/ml) (Figure 3c). In order to verify the general responsiveness of disc cells to the chosen treatment conditions, we also measured changes in MMP13 expression. We found that after 18 hour, treatment with IL-1β (100 ng/ml) resulted in a 146.4 ± 28.0 fold increase of MMP13 expression. Similarly, LPS (2.0 μg/ml) caused an 11.1 ± 2.2 fold increase and TNF-α (100 ng/ml) a 134.0 ± 31.5 fold increase in MMP13 mRNA levels (Mean ± SEM, all $p < 0.001$)(data not shown).

Trichostatin A (a HDAC inhibitor) did not cause any changes in MMP28 expression in human IVD cells at any concentration (18 hours only) (Figure 4a). However, in HeLa cells, which were used as a positive control, Trichostatin A caused a significant 2.1 ± 0.1 fold induction of MMP28 expression at 1000 nM ($p < 0.001$)(data not shown).

Discussion

Our results indicate that MMP28 is expressed by human intervertebral disc cells in vivo and in vitro, with high donor-donor variations in vivo but did not depend on the level of disc degeneration as measured by Thompson grade score. Additionally, we were able to demonstrate that inflammatory cues (LPS, IL-1β and TNF-α) did not regulate the expression of MMP28 in vitro, indicating that inflammatory processes during IVD disease do not seem to regulate MMP28 expression in vivo.

In our study, MMP28 was expressed in most disc samples with overall more pronounced expression in virtually non-degenerated (grade II), traumatic tissue (removed for the need of spinal fusion after trauma) and severely degenerated IVD tissue. However, for both, non-degenerated tissue and the severe degeneration group, high donor-donor variation was observed. Differences in expression levels in similarly degenerated discs suggest that individual processes during degeneration rather than the degeneration stage itself causes an up-regulation of MMP28. In a study done by Gruber et al., MMP28 was measured on the gene expression level using Affymetrix gene array as well as on the protein level using immunohistochemistry on discs with Thompson grade I to IV [19]. Protein detection of MMP28 expression was also anticipated in our study, but commercially available antibodies proved to be unspecific when performing immunoblotting experiments (data not shown). Comparable to our study, Gruber et al. demonstrated that gene expression of MMP28-precursor (gene identification number AF219624.1) tended to be highest in Thompson grade I and II trauma discs and also elevated in severely degenerated and herniated discs, again without any statistical correlation. Therefore, it is still unclear to

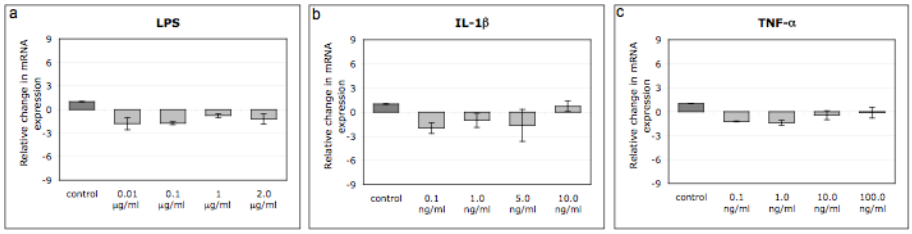

Figure 2 Regulation of MMP28 gene expression in human IVD cells treated with different concentrations of LPS (a), IL-1β (b) or TNF-α (c) for 18 hours (n = 5 each). Data is presented as mean ± SEM; * if $p < 0.05$ between indicated groups.

date whether and how disc diseases can influence MMP28 expression levels. However, increased levels of MMP28 could be detected in cartilage from osteoarthritis and rheumatoid arthritis patients, suggesting that this novel MMP plays a certain, not completely understood role in some musculoskeletal diseases [18,20,21]. So far, it is not clear why some trauma patients showed high MMP28 expression, but it has been described that certain MMPs such as MMP1 may also increase in disc tissue after traumatic incidences [31,32]. The molecular mechanisms underlying the peculiar expression of MMP28 during trauma and certain cases of more severe degeneration is not clear yet and will have to be analyzed further. During degeneration and trauma, specific molecular events may take place, such as apoptotic or inflammatory processes, changes in matrix protein composition (e.g. increase in collagen type I) and alterations in the mechanical environment [32-36], all of which may explain MMP28 regulation. Aside from MMP28, we also measured MMP13 expression, whose levels have been described in the literature to be elevated with degeneration [30,31]. In our samples, we also found a significant and relatively high increase of MMP13 expression in the grade V degeneration group, compared to all lower grades of degeneration, thus confirming previously published data.

However, when testing whether inflammation regulates MMP28 expression, we could not find any changes in MMP28 mRNA levels after treatment with LPS, IL-1β or TNF-α, although inflammatory mediators regulate many other MMPs (e.g. MMP1, MMP3, MMP9, MMP13), as shown in the literature [7-10]. Indeed, when measuring changes in MMP13 expression in our samples, we were able to detect a significant increase after stimulation with all three agents (LPS, IL-1β or TNF-α). This clearly indicates that the absence of MMP28 regulation observed in this study is not due to lack of sensitivity of our model system. As effects on gene expression after stimulation can depend strongly on the used concentrations as well as on the chosen time point for analysis, variations in dose and sampling points were considered in this study, yet no effects were observed under any condition. In human keratinocytes, TNF-α induced MMP28 at least to a minor degree (up to 8 fold), while multiple other growth factors (bFGF, EGF, GM-CSF, HGF, KGF, PDGF, TGF-β1, VEGF, IGF-1) and cytokines (IFN-γ, IL-1β) did not influence its expression levels at all [37]. All this data indicates that compared to other MMPs, MMP28 seems to be rather unresponsive to external inflammatory stimuli in disc cells, although being expressed in degenerative diseases that are characterized by inflammation.

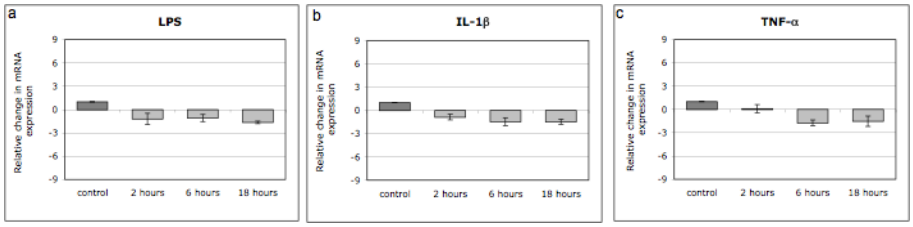

Figure 3 Regulation of MMP28 gene expression in human IVD cells treated with 1 µg/ml LPS (a), 5 ng/ml IL-1β (b) or 10 ng/ml TNF-α (c) for 2, 6 or 18 hours (n = 5 each). Data is presented as mean ± SEM; * if $p < 0.05$ between indicated groups.

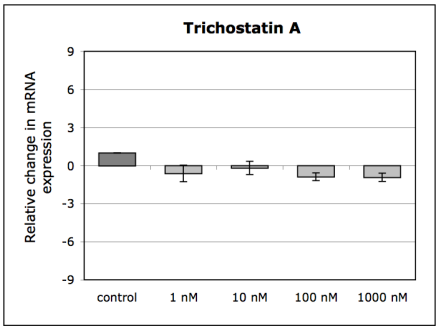

Figure 4 Regulation of MMP28 gene expression in human IVD cells treated with different concentrations of the HDAC inhibitor trichostatin A for 18 hours (n = 3). Data is presented as mean ± SEM; * if $p < 0.05$ between indicated groups.

It should however be noted that, in this part of the study, no distinction was made between annulus fibrosus and nucleus pulposus cells as a clear separation of the two zones is not possible in later stage degenerated disc tissue (whereas a separation was possible in less degraded tissue in the first part). Considering the fact that no effect was observed in this mixed cell population, it is however unlikely that a significant alteration would have been observed if distinct cell types had been used.

As TNF-α was not able to induce MMP28 in human IVD cells, we investigated the potential of trichostatin A, a HDAC inhibitor, which was previously shown to strongly regulate MMP28 in HeLa cells. It is assumed that HDAC inhibitors induce MMP28 promoter by acetylation of specificity protein 1 (SP1), which can alter protein-protein interactions and can modify the SP1 containing protein complexes that act at the GC/GT-boxes [28]. However, in our experiments, trichostatin A did not have any effect on the expression levels of MMP28 in disc cells, but the stimulatory effect in HeLa cells could be confirmed in our experimental setting. So far, no other studies have been performed concerning the responsiveness of MMP28 to HDAC inhibitors. Therefore, it is unknown whether most other cell types would show a behavior similar to HeLa cells (regulation) or to IVD cells (no regulation).

Conclusions

In conclusion, findings of this study provide evidence that MMP28 expression in human IVD tissue is higher in certain cases but the causal relationship between disc diseases and MMP28 expression is unclear to date. In contrast to many other MMPs, MMP28 is not regulated by various inflammatory mediators (IL-1β, TNF-α, LPS) or the HDAC inhibitor trichostatin A. Future studies will be necessary to identify the role of MMP28 in the IVD more conclusively.

Abbreviations

HDAC: histondeacetylase; IL-1β: interleukin-1β; IVD: intervertebral disc; MMP: matrix metalloproteinase; TNF-α: tumor necrosis factor-α.

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Authors' contributions

MK and LQ participated in carrying out the cell culture studies, performing the statistical analysis and drafting the manuscript. AB and MM participated in carrying out the analysis of disc biopsies, performing the statistical analysis and corrected the manuscript.
JS participated in carrying out the analysis of disc biopsies, performing the statistical analysis and corrected the manuscript. Additionally, JS participated in the study design and helped coordinating the study. AGN participated in the study design, contributed in data interpretation and corrected the manuscript. JK provided disc biopsies as well as clinical input and corrected the manuscript. NA provided disc biopsies and obtained funding for part of the study. NB participated in the study design, contributed in data interpretation, obtained funding for part of the study and corrected the manuscript. KW is responsible for the study design, coordinated the study, obtained funding and was responsible for writing the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Triptolide exhibits anti-inflammatory, anti-catabolic as well as anabolic effects and suppresses TLR expression and MAPK activity in IL-1β treated human intervertebral disc cells

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Abstract
Introduction Increased levels of proinflammatory cytokines seem to play a pivotal role in the development of back pain in a subpopulation of patients with degenerative intervertebral disc (IVD) disease. As current treatment options are mostly limited to surgical interventions or conservative treatment, anti-inflammatory substances might offer a novel, more target-orientated therapeutic approach. Triptolide (TPL), a natural substance found in the Chinese medicinal herb Tripterygium wilfordii Hook, has been demonstrated to possess anti-inflammatory effects in various cells, but no studies exist so far for the IVD. Therefore, the aim of this study was to determine the effects of TPL on human IVD cells by analyzing changes in gene expression and underlying molecular mechanisms.
Materials and methods In order to investigate the anti-inflammatory, anabolic and anti-catabolic effect of TPL, dose-dependency experiments ($n = 5$) and time course experiments ($n = 5$) were performed on IL-1β prestimulated human IVD cells and changes in gene expression of IL-6/-8, TNF-α, PGE2S, MMP1/2/3/13, aggrecan and collagen-I/-II were analyzed by real-time RT-PCR. The molecular mechanisms underlying the effects observed upon TPL treatment were investigated by analyzing involvement of Toll-like receptors TLR2/4 (real-time RT-PCR, $n = 5$), NF-κB, MAP kinases p38, ERK and JNK (immunoblotting and immunocytochemistry, $n = 4$) as well as RNA polymerase II (immunoblotting, $n = 3$).
Results Results showed that 50 nM TPL exhibited an anti-inflammatory, anti-catabolic and anabolic effect on the mRNA level for IL-6/-8, PGE2S, MMP1/2/3/13, aggrecan, collagen-II and TLR2/4, with most pronounced changes after 18 h for proinflammatory cytokines and MMPs or 30 h for TLRs and matrix proteins. However, we also observed an up-regulation of TNF-α at higher concentrations. The effects of TPL did not seem to be mediated via an inhibition of NF-κB or a decrease of RNA polymerase II levels, but TPL influenced activity of MAP kinases p38 and ERK (but not JNK) and expression of TLR2/4.
Conclusions In conclusion, TPL may possess promising potential for the treatment of inflammation-related discogenic back pain in vitro, but its analgetic effect will need to be confirmed in an appropriate in vivo animal model.

Keywords Intervertebral disc · Triptolide · Gene expression · Immunoblotting · Signaling pathway

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Introduction

Tripterygium wilfordii Hook F, a vine native to several Asian countries, has been widely used in the traditional Chinese medicine. Triptolide (TPL), a diterpenoid triepoxide, was identified as its major active component. It has been demonstrated to possess strong immunosuppressive and anti-inflammatory effects [1, 2]. TPL has been used in treating inflammatory joint diseases [3, 4], but could potentially also be applied in other inflammation-related diseases. As TPL has been additionally shown to possess anti-proliferative and pro-apoptotic activity in various types of cancer cells, it is considered a possible new candidate in the group of new cancer therapeutics [5].

Current literature on the intervertebral disc (IVD) provides clear evidence for the relevance of inflammatory mediators in the development of back pain. Nucleus pulposus (NP) tissue has long been known to induce radiculopathic pain, due to chemical irritation of dorsal root ganglion nerves induced by proinflammatory cytokines present in this tissue [6–10]. Similarly, degenerative disc disease (leading to so-called discogenic back pain) seems to correlate with increased levels of proinflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α) [11–15]. Although basic knowledge on the increased expression of inflammatory mediators in certain cases of back pain (i.e. discogenic back pain, NP mediated back pain) exists, the molecular mechanisms underlying these processes are not yet elucidated. However, molecular biological research of the past decades indicates that the MAP kinase pathways as well as the transcription factor nuclear factor-κB (NF-κB) are the major mechanisms regulating inflammatory responses in vivo and in vitro. Additionally, TPL has been described to not only influence NF-κB [16–20] and MAP kinases [17, 18, 21], but also to reduce levels of RNA polymerase II (=enzyme that is essential in the process of transcription) in cancer cells [5], thus possibly explaining the broad spectrum of genes whose expression is influenced upon TPL treatment.

In order to suppress inflammation, corticosteroidal substances are frequently used, which are, however, known to have a significant risk for side effects. There is thus increased interest in alternative substances that possess a strong anti-inflammatory and anti-catabolic potential. In the present study, we examined the effects of TPL on the inflammatory and catabolic response of human IVD cells that were prestimulated with recombinant IL-1β to simulate an inflammatory situation. We also analyzed the molecular mechanisms mediating the inhibition of cytokine expression by investigating the role of the transcription factor nuclear factor kappa B (NF-κB), the Toll-like receptors TLR2 and TLR4, the mitogen-activated protein (MAP) kinases including p38, ERK (=extracellular signal-regulated kinase) and JNK (=c-Jun N-terminal kinase) as well as the involvement of RNA polymerase II.

Materials and methods

Materials

Materials used in this study are specified in Table 1.

Human intervertebral disc cell culture

Human IVD tissue was removed from patients undergoing spinal surgery for discectomy or interbody fusion for degenerative disc disease or disc herniation (for detailed information see Table 2) after obtaining informed consent in accordance with the local ethical guidelines.

Table 1 Detailed information about materials used in this study

Material	Supplier
Ampicillin	Gibco
BSA	Sigma-Aldrich
Bradford reagent	Bio-Rad
Collagenase NB4	Serva
Dispase II	Roche
DMSO	Sigma
ERK/p-ERK antibodies	Cell Signaling
F-12/DMEM medium	Sigma-Aldrich
Fetal calf serum (FCS)	Tecomedical
HRP-mouse antibody	Amersham
HRP-rabbit antibody	Sigma
Hyperfilm ECL	Amersham
Hybond-P PVDF	Amersham
IL-1β recombinant	Peprotech
JNK/p-JNK antibodies	Cell Signaling
MTT	Sigma-Aldrich
p38/p-p38 antibodies	Cell Signaling
p65 antibody	Santa Cruz
PARP1 antibody	Santa Cruz
PCR Master Mix	Applied Biosystems
Penicillin	Gibco
PureLink RNA Kit	Invitrogen
Reverse transcription reagents	Applied Biosystems
RNA polymerase II antibody	Santa Cruz
Streptomycin	Gibco
SuperSignal West Dura	Socochim
Triptolide	Sigma
Trypsin	Invitrogen
Tubulin antibody	Cell Signaling

Table 2 Demographic data on surgical disc samples used in this study

Number	Age	Sex	Pathology	Grade	Level
1	60	M	Disc herniation	3	L4/5
2	51	F	Sympt. disc disease	4	L5/S1
3	42	F	Disc herniation	4	L4/5
4	50	M	Disc herniation	4	L4/5
5	47	M	Disc herniation	4	L4/5
6	46	F	Disc herniation	3	L5/S1
7	54	M	Disc herniation	4	L4/5
8	27	F	Disc herniation	3	L4/5
9	26	F	Disc herniation	3	L4/5
10	43	M	Disc herniation	4	L4/5
11	43	F	Disc herniation	4	L4/5
12	50	F	Disc herniation	3	L5/S1
13	49	M	Disc herniation	4	L5/S1
14	26	M	Disc herniation	4	L5/S1
15	45	F	Disc herniation	4	L5/S1
16	40	F	Disc herniation	4	L4/5
17	57	M	Disc herniation	4	L4/5
18	44	F	Disc herniation	3	L5/S1
19	60	M	Disc herniation	4	L4/5
20	47	M	Disc herniation	4	L4/5
21	36	M	Disc herniation	3	C5/6
22	53	M	Disc herniation	4	L4/5
23	48	F	Disc herniation	4	L4/5
24	48	F	Disc herniation	3	L4/5
25	42	F	Disc herniation	4	L4/5
26	60	M	Sympt. disc disease	3	L4/5
27	46	F	Disc herniation	4	L5/S1
28	27	F	Disc herniation	4	L4/5
29	42	M	Disc herniation	3	L4/5

M male, *F* female, *grade* classification of intervertebral disc degeneration by Pfirrmann grade

IVD tissue was enzymatically digested (0.2% collagenase NB4, 0.3% dispase II) for 4–8 h and cells were thereafter cultured in DMEM/F12 supplemented with 10% FCS, penicillin (50 units/ml), streptomycin (50 µg/ml) and ampicillin (125 ng/ml) up to passage 2–3.

Viability measurement

Cellular viability after treatment with different concentrations of TPL (6, 18 and 30 h) was analyzed using the MTT assay as previously described, which has also been shown to be comparable to other viability/toxicity measurements (Picogreen assay, cell counting) [22]. Non-toxic concentrations were chosen for subsequent experiments.

Gene expression analysis

In order to investigate the effects of TPL on the expression of inflammatory mediators (IL-6, IL-8, TNF-α, PGE2S = prostaglandin E2 synthase), matrix degrading enzymes (MMP1, MMP2, MMP3, MMP13), Toll-like receptors (TLR2, TLR4) and anabolic genes (aggrecan collagen-I, collagen-II), dose-dependency (0.5, 5, 50 nM—all 18 h) and time course experiments (6, 18, 30 h—all 50 nM) were performed. Cells were serum starved for 2 h and then exposed to 5 ng/ml IL-1β for 2 h before treating cells with different concentration of TPL for 18 h. The most active concentration was chosen for further time course experiments. Untreated control cells as well as DMSO treated cells (=solvent) were included in each experiment. After treatment, cells were harvested by trypsin, RNA was isolated, reverse transcribed and gene expression was measured on the StepOne Plus PCR machine (Applied Biosystems) using real-time RT-PCR as previously described. Briefly, human specific probes and primers (see Table 3), TaqMan real-time RT-PCR Mix and cDNA were mixed, measured in duplicates and data was analyzed by using the comparative ct method ($2^{-\Delta\Delta C_t}$ method, housekeeping gene = Tata Box binding protein = TBP). The assay was performed on samples from five independent experiments.

Table 3 Primers/probes used for real-time RT-PCR (TaqMan® Gene Expression Assays, Applied Biosystems)

Gene	Primer sequence number	Base pairs
Aggrecan	Hs00202971_m1	93
Collagen-I	Hs00164004_m1	66
Collagen-II	Hs00264051_m1	124
Interleukin-6 (IL-6)	Hs00174131_m1	95
Interleukin-8 (IL-8)	Hs00174103_m1	101
Matrixmetalloproteinase-1 (MMP1)	Hs00233958_m1	133
Matrixmetalloproteinase-2 (MMP2)	Hs00174131_m1	96
Matrixmetalloproteinase-3 (MMP3)	Hs01548724_m1	98
Matrixmetalloproteinase-13 (MMP13)	Hs00233992_m1	91
Prostaglandin E2 Synthase (PGE2S)	Hs00228159_m1	66
Tata Box binding protein (TBP)	Hs00427620_m1	91
Toll-like receptor 2 (TLR2)	Hs00152932_m1	80
Toll-like receptor 4 (TLR4)	Hs00152939_m1	89
Tumor necrosis factor α (TNF-α)	Hs00174128_m1	80

Pathway analysis (NF-κB, MAP kinases)

In order to investigate potentially involved pathways, immunoblotting was performed for p65 (subunit of NF-κB) as well as for the phosphorylated (=activated) and unphosphorylated MAP kinases p38, ERK and JNK. Briefly, cells were stimulated with IL-1β or co-stimulated with IL-1β and 50 nM of TPL (the most potent concentration in gene expression experiments) for 15 min (MAP kinases) or 60 min (NF-κB). Unstimulated control cells were included as well.

For NF-κB immunoblotting, nuclear extracts were prepared according to standard protocols, protein content was measured by Bradford assay, nuclear extracts were fractioned by SDS-PAGE, proteins were transferred onto membranes and first incubated with a p65 antibody and then with the appropriate secondary HRP-labeled antibody before analyzing chemiluminescence. In order to confirm NF-κB immunoblotting results, nuclear translocation of p65 was additionally examined by immunocytochemistry in methanol-fixed cells using standard techniques.

For MAP kinase immunoblotting, whole cell extracts were prepared as previously described, protein content was measured by Bradford assay, whole cell extracts were fractioned by SDS-PAGE, proteins were transferred onto membranes and first incubated with antibodies recognizing phosphorylated or unphosphorylated p38, ERK (=p42/44) or JNK antibody and then with the appropriate secondary HRP-labeled antibody before analyzing chemiluminescence.

PARP1 and tubulin were used as loading controls for p65 and MAP kinase immunoblotting, respectively. Each assay was performed on samples from four independent experiments.

Analysis of RNA polymerase II protein expression level

The effect of TPL on RNA polymerase II expression was investigated by immunoblotting of whole cell extracts that were obtained from cells stimulated with either IL-1β alone or IL-1β prestimulated cells with 50 nM of TPL for 6, 18 or 30 h (see "Gene expression analysis"), using a specific antibody recognizing human RNA polymerase II. Tubulin was used as a loading control and the assay was performed on samples from three independent experiments.

Statistical analysis

Statistical analysis was performed by Mann–Whitney *U* Test (two-tailed) using the SPSS software. A significance level of $P < 0.05$ was considered statistically significant.

Results

Viability

TPL exhibited a cytotoxic effect at concentrations of 250 nM and higher already within 18 h (data not shown). For further experiments, non-toxic concentrations of 0.5, 5 and 50 nM were chosen, which did not exhibit any statistically significant cytotoxic effect within the time frame of the subsequent experiments (up to 30 h).

Gene expression

Confirming our data from previous studies, pre-treatment with IL-1β stimulated expression of IL-6, IL-8, TNF-α,

Table 4 Effects of IL-1β stimulation on mRNA levels of candidate genes after 6, 18 and 30 h, indicated as fold change relative to no treatment (mean, SEM, *P* values)

Gene	Time point (h)	Mean fold change	SEM	*P* value
Aggrecan	6	−1.4	0.3	0.001
	18	−1.8	0.5	0.001
	30	−2.7	0.5	<0.0001
Collagen-I	6	−1.0	0.2	0.001
	18	−1.3	0.5	0.015
	30	−2.1	0.2	<0.0001
Collagen-II	6	−2.1	0.2	<0.0001
	18	−8.9	3.2	<0.0001
	30	−7.2	1.1	<0.0001
IL-6	6	432.7	74.3	<0.0001
	18	6236.1	2601.3	<0.0001
	30	7350.5	2557.3	<0.0001
IL-8	6	558.4	175.2	<0.0001
	18	1102.5	291.3	<0.0001
	30	1983.8	649.3	<0.0001
MMP1	6	449.1	163.9	<0.0001
	18	1576.5	470.9	<0.001
	30	2032.6	631.9	<0.0001
MMP2	6	0.7	0.3	0.419
	18	2.1	0.5	<0.0001
	30	3.4	0.3	<0.0001
MMP3	6	327.7	124.9	<0.0001
	18	1143.5	240.7	<0.0001
	30	1768.8	636.0	<0.0001
MMP13	6	79.1	20.6	<0.0001
	18	114.6	30.2	<0.0001
	30	297.3	140.0	<0.0001
TNF-α	6	78.5	16.4	<0.0001
	18	41.1	14.4	<0.0001
	30	46.0	11.3	<0.0001
TLR2	6	16.5	1.9	<0.0001
	18	13.5	1.2	<0.0001
	30	13.4	2.1	<0.0001

Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta C_t}$ method) ($n = 5$). PGE2S and TLR4 were not significantly regulated by IL-1β stimulation (values not shown)

MMP1, MMP2, MMP3, MMP13 as well as TLR2 and reduced expression of aggrecan, collagen-I and collagen-II (for detailed results, see Table 4), but did not influence expression of PGE2S and TLR4.

In the first set of experiments, IL-1β prestimulated cells were treated with different concentrations of TPL (0.5, 5, 50 nM) for 18 h. We observed a dose-dependent inhibition of the expression of inflammatory mediators (IL-6, IL-8, PGE2S), matrix degrading enzymes (MMP1, MMP2, MMP3, MMP13) and Toll-like receptors (TLR2, TLR4). For aggrecan, a 1.9-fold increase was observed with 50 nM after 18 h, while no changes occurred with the lower concentrations. Collagen-I and collagen-II were either not altered or slightly decreased after 18 h. For all results, see Fig. 1a–d. TNF-α expression was increased at concentrations of 5 nM (2.8-fold) and 50 nM (21.2-fold) (data not shown).

For the second set of experiments, IL-1β prestimulated cells were treated with 50 nM TPL for 6, 18 or 30 h (time course experiments). Results show that TPL exhibits its anti-inflammatory, anti-catabolic and anabolic effects already after 6 h with regard to IL-6, IL-8, MMP1, MMP3, MMP13, aggrecan and TLR2, but its effects were more pronounced after longer incubation periods (18 and 30 h), including an increase of collagen-II (Fig. 2a–d). The most distinct reduction in gene expression was observed at 18 h for IL-6 (100 → 4.1%), MMP1 (100 → 11.1%), MMP3 (100 → 15.7%), MMP13 (100 → 13.3%) and TLR4 (100 → 18.2%), but effects were also significant for IL-8 (100 → 36.5%), MMP2 (100 → 42.2%), PGE2S (100 → 26.4%)

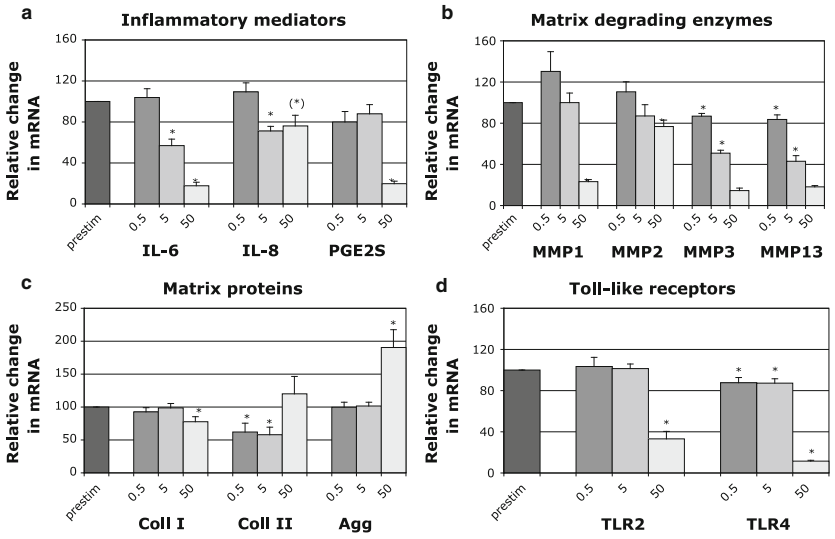

Fig. 1 Effects of different concentrations of TPL (0.5, 5, 50 nM—18 h) on mRNA levels of candidate genes, indicated as fold change relative to IL-1β-prestimulation (set to 100%): **a** inflammatory mediators (IL-6, IL-8, PGE2S), **b** matrix degrading enzymes (MMP1, MMP2, MMP3, MMP13), **c** matrix proteins (aggrecan, collagen-I, collagen-II) and **d** Toll-like receptors (TLR2, TLR4). Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta C_t}$ method) and is presented as Mean and SEM ($n = 5$). *Asterisks* indicate statistical significance ($P < 0.05$); an *asterisk in brackets* indicates barely significant (IL-8: $P = 0.051$)

and TLR2 (100 → 30.1%). We observed a time-dependent up-regulation of aggrecan with a significantly increase after 18 h (1.8-fold), but the effect was more pronounced after 30 h (2.7-fold). Similarly, collagen-II expression was significantly induced after 30 h (3.1-fold), while collagen-I expression was not altered at any time point. While matrix proteins were most regulated after 30 h, effects declined for several genes at this time point already. Nevertheless, the inhibitory effect remained significant for IL-6, MMP1, MMP2, MMP13, PGE2S, TLR2 and TLR4 (Fig. 2a–d). In accordance with results of the dose-dependency experiments, TNF-α expression was up-regulated, especially after 30 h (6 h: 4.8-fold; 18 h: 101.0-fold; 30 h: 987.3-fold) (data not shown).

NF-κB and MAP kinase pathway

Immunoblotting for p65 indicates that IL-1β prestimulation caused nuclear translocation of p65, which is the first step of NF-κB activation. However, treating IL-1β prestimulated cells with 50 nM TPL was not able to prevent or reverse nuclear translocation of NF-κB. Figure 3a shows that the p65 band of TPL treated samples is not reduced compared to IL-1β stimulated samples, while untreated cells show a much smaller amount of target protein as detected by immunoblotting of nuclear extracts. Equal protein loading was confirmed by PARP1 detection. This pattern could be confirmed by immunocytochemistry as shown in Fig. 3b.

Immunoblotting for MAP kinases indicated that IL-1β prestimulation caused phosphorylation of p38, ERK and JNK, which is indicative of their activation. TPL treatment (50 nM) strongly reduced levels of phosphorylated p38 (Fig. 3c) and slightly reduced levels of phosporylated ERK (Fig. 3d), but not of JNK (Fig. 3e) compared to IL-1β stimulated samples. As expected, levels of unphosporylated p38, ERK and JNK were similar in all groups. Equal protein loading was confirmed by tubulin detection.

RNA polymerase II protein expression level

Immunoblotting for RNA polymerase II indicates that 50 nM TPL did not influence its expression levels at any investigated time point (6, 18, 30 h), compared to IL-1β stimulated samples or untreated samples (Fig. 4).

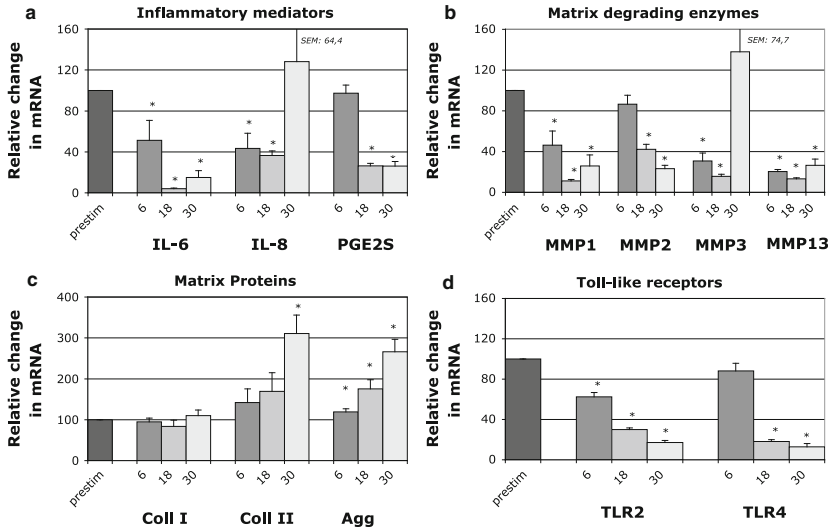

Fig. 2 Effects of TPL (50 nM) on mRNA levels of candidate genes after different time points (6, 18, 30 h), indicated as fold change relative to IL-1β-prestimulation (set to 100%): **a** Inflammatory mediators (IL-6, IL-8, PGE2S), **b** matrix degrading enzymes (MMP1, MMP2, MMP3, MMP13), **c** matrix proteins (aggrecan, collagen-I, collagen-II) and **d** Toll-like receptors (TLR2, TLR4). Data was obtained by real-time RT-PCR ($2^{-\Delta\Delta C_t}$ method) and is presented as Mean and SEM ($n = 6$). *Asterisks* indicate statistical significance ($P < 0.05$)

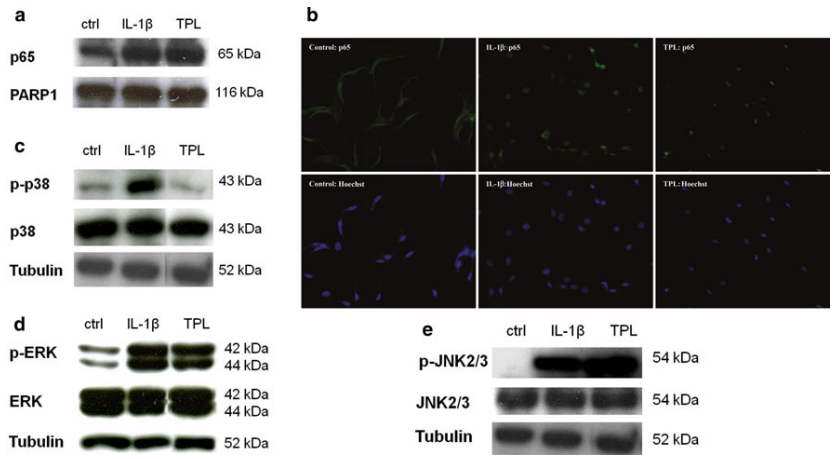

Fig. 3 Effects of TPL (50 nM) on the induction/activity of NF-κB and MAP kinases, detected by immunoblotting (IB) and immunocytochemistry (IC). NF-κB induction was detected by **a** IB of nuclear extracts ($n = 4$, 60 min) and **b** IC ($n = 4$, 60 min). MAP kinase activity was detected by IB of whole cell extracts ($n = 4$, 15 min) for **c** p38, **d** ERK and **e** JNK. One representative sample is shown. For IB, PARP1 or tubulin was used as a loading control. For IC, Hoechst counterstaining of cell nuclei was performed

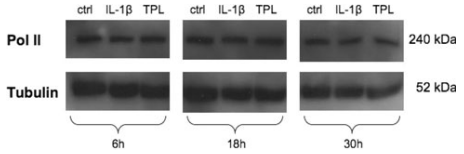

Fig. 4 Effects of TPL (50 nM) on the expression levels of RNA polymerase II, detected by immunoblotting of whole cell extracts ($n = 3$) (6, 18, 30 h). One representative sample is shown. Tubulin was used as a loading control

Discussion

Tripterygium wilfordii Hook has its therapeutic origin in the traditional Chinese medicine (TCM), which—during the past years—has been recognized by researchers worldwide as an important and extensive source for revealing novel lead molecules for modern drug discovery. However, typical TCM preparations usually combine multiple herbs (or other natural substances) with the respective variability in composition (e.g. due to harvesting periods) and can thus not be regarded as standardized products. In order to create patentable and marketable products for conventional Western medicine, the active component has to be identified and its molecular mechanism of action should be elucidated. TPL has been identified as one of the major bioactive components of Tripterygium wilfordii Hook. The therapeutic potential of TPL or Tripterygium wilfordii Hook is currently investigated at the stage of clinical trials for the treatment of polycystic kidney disease, glomerulosclerosis, nephropathy, spondylitis and rheumatoid arthritis (http://clinicaltrials.gov). On a laboratory stage, the anti-inflammatory and anti-catabolic effects of TPL are currently being investigated in even more cell and tissue types, thus trying to broaden its therapeutic use. However, to the authors' knowledge, no studies have been performed with regard to IVD diseases yet.

Results from this study on human IVD cells indicate that TPL can effectively reduce mRNA levels of major inflammatory mediators (IL-6, IL-8, PGES2) and matrix degrading enzymes (MMP1, MMP2, MMP3, MMP13), with highest effects after 18 h. However, mRNA expression of TNF-α was up-regulated upon TPL treatment. Additionally, 50 nM TPL significantly induced levels of relevant matrix proteins (aggrecan, collagen-II), especially after 30 h. Why lower concentrations of TPL seemed to inhibit collagen-II expression at earlier time points (18 h) is currently not clear. While matrix proteins responded strongly to TPL treatment after 30 h, effects were not as pronounced anymore at this time point for most inflammatory and catabolic genes, indicating that TPL may possess a limited bioactivity time frame. However, usage of a slow release system may overcome this restriction. Pathway analysis provides evidence that this effect may be (at least partially) mediated by the MAP kinases p38 and ERK (whose activity we saw to be influenced by TPL), while the transcription factor NF-κB, the MAP kinase JNK or the RNA polymerase II did not seem to be involved in signal transduction. Additionally, expression of Toll-like receptors TLR2 and TLR4 was reduced by TPL treatment.

The observed inhibition of mRNA for the major matrix degrading enzymes and the induction of matrix proteins in human IVD cells suggests that TPL can block tissue degradation and may thus potentially slow down or prevent further disc degeneration. A comparable anti-catabolic potential of TPL has been described in other cell types before, e.g. TPL was shown to inhibit expression of proMMPs 1 and 3 in human synovial fibroblasts [23] and expression of MMP3, MMP13 and ADAMTS4 in human OA chondrocytes [24].

In the present in vitro study, TPL was able to inhibit inflammatory responses in human IVD cells, which is similar to macrophages (inhibition of PGE2, IL-1α, IL-β and IL-6 expression) [23] and various kinds of fibroblasts (inhibition of PGE2, COX-2, IL-6 and IL-8 expression) [20, 25–27]. Although to the author's knowledge only few studies exist to date that investigated the anti-inflammatory in vitro effect of TPL in chondrocytes [24], its effects were tested in a collagen-induced arthritis mouse model in vivo. Results indicate that TPL can reduce inflammatory responses and cartilage damage in the joint tissues by inhibiting expression of MMP3, MMP13, PGE2, COX-2, IL-1β, IL-6, TNF-α [16–18]. While we can confirm the inhibition of MMP3, MMP13 and IL-6 in human IVD cells in vitro, expression of TNF-α was induced in our system and will thus be subject to further scrutiny in next experiments. The increased level of TNF-α may not only be critical for any in vivo application, but may have also influenced our results if transferred to the protein level as it may be responsible for the sustained and unaltered NF-κB activation. Activation of NF-κB has been described to cause an anti-apoptotic effect, thus possibly masking any toxic effect of TPL [28]. However, in order to verify whether this is the case, experiments with NF-κB inhibition (e.g. using Ad5-I kappa B alpha Delta N; MG132 [28]) will need to be performed in the future. This will be important as in tumor cells, TPL could in contrast block TNF-α-induced activation of NF-κB, resulting in enhanced apoptosis induced by TNF-α [29].

Based on the obtained results, the next step will be to investigate the anti-inflammatory and analgetic behavior of TPL in vivo, using a well-established rodent model of radiculopathic pain [30]. As bioavailability and diffusion rates in vivo are unclear, the in vitro data obtained in this study can only provide a first basis to choose appropriate in vivo application modes.

Based on the promising gene expression results, we further sought to investigate the underlying molecular mechanism. Findings in the literature indicate that the NF-κB pathway is one of the molecular mechanisms underlying the cellular responses observed after TPL treatment [16–20]. However, the effects of TPL seem to be mediated by a complex interplay of various signaling mechanisms, including the MAP kinases p38 [17], ERK [21] or JNK [17, 18] as well as the transcription factor AP-1 [19]. We were able to show that in IVD cells, TPL may work in part by interfering with phosphorylation of the MAP kinases p38/ERK and by regulating expression of TLR2/4, but probably not by inhibiting NF-κB or JNK activity, although this is described for multiple other cell types [16–20], indicating that the involved signaling pathways are cell-specific. In addition, we investigated whether TPL is able to influence protein levels of RNA polymerase II as this has been described to be a major mechanism of action of TPL in cancer cells most recently [5]. However, in human disc cells, TPL did not reduce RNA polymerase II levels as shown by immunoblotting, indicating once more that TPL acts cell-specifically on certain mechanisms/pathways.

Clinical relevance

TPL seems to be a promising candidate to treat certain cases of discogenic or NP mediated back pain, in which increased levels of proinflammatory cytokines are responsible for pain sensation. The in vitro cell culture results clearly showed that TPL could effectively inhibit several proinflammatory cytokines and matrix degrading enzymes, which are thought to play a major role during symptomatic disc degeneration. Simultaneously, TPL induced expression of disc-specific matrix proteins. However, in vivo experiments will be needed to verify that TPL is an attractive, new therapeutic agent for degenerative disc disease by preventing further degradation of the tissue and exhibiting an analgetic effect due to inhibition of proinflammatory cytokines.

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Conflict of interest None.

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BASIC SCIENCE

The Red Wine Polyphenol Resveratrol Shows Promising Potential for the Treatment of Nucleus Pulposus–Mediated Pain *In Vitro* and *In Vivo*

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Study Design. Descriptive and mechanistic investigation of the anti-inflammatory and anticatabolic effect of resveratrol in intervertebral discs (IVDs) *in vitro* and of the analgetic effect *in vivo*.

Objective. To determine whether resveratrol may be useful in treating nucleus pulposus (NP)–mediated pain.

Summary of Background data. Proinflammatory cytokines seem to be key mediators in the development of NP-mediated pain. Patients with discogenic or radiculopathic pain may substantially benefit from anti-inflammatory substances that could be used in a minimal-invasive treatment approach. Resveratrol, a polyphenolic phytoalexin found in red wine exhibits anti-inflammatory effects in various cell types and tissues, but no data exists so far with regards to the IVD in the context of low back and leg pain.

Methods. In part 1, the anti-inflammatory and anticatabolic effect of resveratrol was investigated in a cell culture model on interleukin 1β (IL-1β) prestimulated human IVD cells on the gene and protein expression level. In part 2, the molecular mechanisms underlying the effects observed upon resveratrol treatment were investigated (toll-like receptors, nuclear factor κB, sirtuin 1 (SIRT1), mitogen-activated protein (MAP) kinases p38/ERK/JNK). In part 3, the analgetic effects of resveratrol were investigated *in vivo* using a rodent model of radiculopathy and von Frey filament testing. All quantitative data were statistically evaluated either by Mann-Whitney *U* test or by one-way analysis of variance and Bonferroni *post hoc* testing ($P < 0.05$).

Results. *In vitro*, resveratrol exhibited an anti-inflammatory and anticatabolic effect on the messenger RNA and protein level for IL-6, IL-8, MMP1, MMP3 and MMP13. This effect does not seem to be mediated via the MAP kinase pathways (p38, ERK, JNK) or via the NF-κB/SIRT1 pathway, although toll-like receptor 2 was regulated to a minor extent. *In vivo*, resveratrol significantly reduced pain behavior triggered by application of NP tissue on the dorsal root ganglion for up to 14 days.

Conclusion. Resveratrol was able to reduce levels of proinflammatory cytokines *in vitro* and showed analgetic potential *in vivo*. A decrease in proinflammatory cytokines may possibly be the underlying mechanism of pain reduction observed *in vivo*. Resveratrol seems to have considerable potential for the treatment of NP-mediated pain and may thus be an alternative to other currently discussed (biological) treatment options.

Key words: anticatabolic, anti-inflammatory, MAP kinases, nucleus pulposus–mediated back pain, NF-κB, radiculopathy, resveratrol, toll-like receptor. **Spine 2011;36:E1372–E1383**

Nucleus pulposus (NP) tissue has long been known to be a potent inducer of pain (*e.g.*, in radiculopathy). Scientific evidence indicated that this effect is due to proinflammatory cytokines present in this tissue.[1–5] Degenerative disc disease (leading to so-called discogenic back pain) also seems to correlate with increased levels of proinflammatory cytokines.[6–12] The treatment of NP-mediated pain such as radiculopathic pain and discogenic pain is still a matter of debate, indicating that current treatment options are not ideal. This is most likely because of the fact that current therapeutic strategies do not account for the biological mechanisms of pain development. There is a clear need for the development of novel therapeutic procedures that have the ability to interfere with the biological mechanisms of NP-mediated pain, that is, with increased expression of pain-mediating proinflammatory cytokines. Injectable anti-inflammatory substances that specifically target the metabolism of intervertebral disc (IVD) cells may serve as new and useful minimal-invasive treatment options,

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either for radiculopathic pain or discogenic back pain. The substance of choice should ideally also exhibit a strong anti-catabolic effect, thus limiting further degeneration of the IVD.

There is a growing interest in natural bioactive compounds that may have a pronounced anti-inflammatory effect and could thus possibly replace corticosteroids. Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin found in red wine that has previously been shown to demonstrate anti-inflammatory, anticancer, immunomodulatory, cardioprotective, antioxidative, and chemopreventive capabilities in various cell and tissue types[13–20]. However, it is unclear whether resveratrol also has an anti-inflammatory effect on IVD cells.

The most commonly regulated pathways/receptors in inflammation are the transcription factor nuclear factor κB (NF-κB), the mitogen-activated protein (MAP) kinase family (p38 MAP kinase [p38], extracellular-signal regulated kinase [ERK], c-Jun N-terminal kinase [JNK]), and the toll-like receptors (TLRs). Anti-inflammatory substances may reduce proinflammatory cytokines on the messenger RNA (mRNA) and/or protein level by inhibiting one or several of these (and/or possibly also alternative) signaling pathways.

On the basis of the obvious needs for more defined, biological treatment options for NP-mediated pain, we aimed to investigate the promising candidate resveratrol *in vitro* and *in vivo*. The detailed aims of this study were the following:

1. To analyze the anti-inflammatory and anticatabolic potential of resveratrol in an *in vitro* IVD cell culture model.
2. To identify the signal transduction mechanisms underlying the observed effects *in vitro*.

TABLE 1. Detailed Information on Chemicals and Antibodies

Chemical	Supplier	Antibody	Supplier
Ampicillin	Gibco	ERK/p-ERK	Cell Signaling
BSA	Sigma-Aldrich	IL-6	Sigma
Bradford reagent	Bio-Rad	IL-8	Genscript
Collagenase NB4	Serva	JNK/p-JNK	Cell Signaling
Dispase II	Roche	MMP1	Genetex
EMSA kits	Panomics	MMP3	Genetex
F-12/DMEM medium	Sigma-Aldrich	MMP13	Genetex
FCS	Tecomedical	PARP-1/2	Santa Cruz
Hyperfilm ECL	Amersham	p38/p-p38	Cell Signaling
Hybond-P PVDF	Amersham	p65	Santa Cruz
IL-1β recombinant	Peprotech	Tubulin	Cell Signaling
Mowiol 4—88	Roth		
MTT	Sigma-Aldrich	HRP-mouse	Amersham
Na-Deoxycholate	Sigma-Aldrich	HRP-rabbit	Sigma
PCR Mater Mix	Applied Biosyst.	CY3 rabbit	Abcam
Penicillin	Gibco		
PureLink RNA Kit	Invitrogen		
Resveratrol	Sigma-Aldrich		
Rev. Transc. Reagents	Applied Biosyst.		
Sirtinol	Sigma-Aldrich		
Sodium Pentobarbital	Abbott		
Streptomycin	Gibco		
SuperSignal West Dura	Socochim		
Trichloro Acetic Acid	Sigma-Aldrich		
Triton-X 100	Sigma-Aldrich		
Trypsin	Invitrogen		

3. To examine the analgetic potential of resveratrol to reduce NP-mediated pain, using an *in vivo* rodent animal model of radiculopathic pain.

MATERIALS AND METHODS

Chemicals and Antibodies

Detailed information on all chemicals and (primary and secondary) antibodies used in this study is given in Table 1.

IVD Cell Isolation and Culture

IVD tissue samples were obtained during spinal surgery (Table 2) after informed consent was granted by the patients. The study was approved by the institutional review board. Disc cells were isolated from the biopsies by enzymatic digestion with 0.3% collagenase NB4 and 0.2% dispase II in phosphate buffered saline (PBS) for approximately 6 hours at 37°C. Primary IVD cells were cultured in growth medium (Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture [DMEM-F12], 10% fetal calf serum [FCS], 50 U/mL penicillin, 50 μg/mL streptomycin, 125 ng/mL ampicillin) and passaged by trypsin treatment at approximately 80% confluency (up to three passages). Biopsies were separated into NP and annulus fibrosus (AF) on the basis of the macroscopic appearance of the disc. Great care was taken that NP tissue was processed for further analysis.

Cell Viability Assay

Nontoxic concentrations of resveratrol that were used in cell culture experiments were determined by the MTT assay. Briefly, cells were cultured in 24-well plates and treated with 11 different concentrations of resveratrol (between 1000 μM

TABLE 2. Demographic Data on Surgical Disc Samples, With Specification of the Experiments That Were Performed With Each Sample (M = Male; F = Female)

Number	Sex	Age	Level	Pathology	Pfirrmann Grade	Experiment*
1	M	54	L2/3	Herniation	1	G
2	M	65	L5/S1	Stenosis/ Protrusion	4	G
3	M	69	L4/5	Herniation	2	G
4	M	38	L4/5	Herniation	3	G
5	F	20	L5/S1	Isthmic Spondylolisthesis	5	G
6	M	59	L4/5	Herniation	1	M
7	M	56	L5/S1	Herniation	4	M
8	F	59	L4/5	Herniation	5	G, P
9	F	45	L2/3	Herniation	3	P
10	F	28	L5/S1	Herniation	2	M
11	M	31	L5/S1	Herniation	3	V, M
12	F	66	L4/5	Herniation	3	V, M
13	F	45	L5/S1	Herniation	5	M
14	F	46	L5/S1	Herniation	3	M
15	M	47	L4/5	Herniation	4	V
16	M	58	L2/3	Herniation/ Stenosis	5	V
17	M	44	L5/S1	Recurrent Herniation	3	V
18	F	45	L5/S1	Herniation	5	V, M
19	M	27	L5/S1	Herniation	4	M
20	F	36	L5/S1	Herniation	2	M
21	F	30	L4/5	Herniation	3	M
22	F	40	L4/5	Herniation	3	M
23	M	56	L4/5	Sequestration	3	P

**V = viability; G = gene expression analysis; P = protein expression analysis; M = mechanistic pathway analysis (all methods).*

TABLE 3. Primers/Probes Used for Real-Time RT-PCR (TaqMan Gene Expression Assays, Applied Biosystems)

Gene	Primer Sequence Number	Base Pairs
TATA box binding protein (TBP)	Hs00427620_m1	91
Interleukin-1β (IL-1β)	Hs00174097_m1	94
Interleukin-6 (IL-6)	Hs00174131_m1	95
Interleukin-8 (IL-8)	Hs00174103_m1	101
Toll-like receptor 2 (TLR2)	Hs00152932_m1	80
Matrixmetalloproteinase-1 (MMP1)	Hs00233958_m1	133
Matrixmetalloproteinase-3 (MMP3)	Hs00968308_m1	98
Matrixmetalloproteinase-13 (MMP13)	Hs00233992_m1	91

and 0 μM, 1:2 dilution each time) for 18 hours before adding a fresh, sterile solution of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) with a concentration of 0.5 mg/mL in DMEM/F12. After 2 to 3 hours at 37°C, MTT was discarded, cells were lysed with DMSO for 10 minutes and absorbance was measured at 565 nm. Absorbance of resveratrol treated cells was calculated relative to absorbance of untreated control cells. The assay was performed in triplicates and the results are provided as mean values with standard error of the mean from six independent experiments.

Analyzing the Effects of Resveratrol on the Gene Expression Level

Expanded cells in passage 2 or 3 were rendered serum free for 2 hours and then preincubated with IL-1β (5 ng/mL) for 2 hours to induce an inflammatory and catabolic response. Thereafter, resveratrol (in EtOH, 5 μM or 50 μM) was added and cells were incubated for additional 18 hours. Untreated control cells as well as EtOH-treated cells were included in the experimental setup. After incubation, mRNA was isolated, 1 μg was reverse transcribed to complementary DNA and gene expression of IL-1β, IL-6, IL-8, MMP1, MMP3, MMP13 and the toll-like receptor TLR2 was measured (primer details are given in Table 3). After normalizing to the housekeeping gene (TBP), which was chosen on the basis of unresponsiveness to the applied treatment, expression of resveratrol treated cells was compared with untreated control or EtOH-treated cells using the $2-\Delta\Delta Ct$ method[21]. The assay was performed in duplicates and the results are provided as mean values with standard error of the mean from six independent experiments.

Analyzing the Effects of Resveratrol on the Protein Expression Level

Cells were treated as described above for gene expression analysis and conditioned media was harvested after 18 hours. Protein was precipitated from the medium by incubation with Na-Deoxycholate for 1 hour, followed by trichloracetic acid over night at 4°C. The pellet was resuspended in RIPA buffer and protein content was measured using Bradford reagent before analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Briefly, samples were denatured and loaded onto a polyacrylamide gel. After separation by electrophoresis, proteins were transferred to a PVDF membrane, blocked in tris-buffered saline tween-20 (TBS-T) with 5% milk-powder (1 hour) and then incubated with a specific antibody for IL-6, IL-8, MMP1, MMP3 and MMP13 (candidates were chosen on the basis of highest effects seen on the mRNA level) at the appropriate dilution for 2 hours. After washing, membranes were incubated with the respective secondary antibody at the appropriate concentration for 1 hour at room temperature. SuperSignal West Dura was used for detection of chemiluminescence on Hyperfilm. The assay was performed on supernatants from three independent experiments.

Analyzing the Effects of Resveratrol on SIRT1 Activity

Expanded cells in passage 2 or 3 were rendered serum free for 2 hours, preincubated with IL-1β (5 ng/mL) and thereafter resveratrol alone (50 μM) or in combination with the SIRT1 inhibitor sirtinol (1, 5 or 10 μM) was added and cells were incubated for additional 18 hours. Untreated control cells as well as ethanol (EtOH) treated cells were included in the experimental setup. After stimulation, gene expression was analyzed as described earlier. The assay was performed in duplicates and the results are provided as mean values with standard error of the mean from six independent experiments.

Immunocytochemistry-Based NF-κB Activation Assay

The effect of resveratrol on the IL-1β–induced nuclear translocation of p65 was examined by immunocytochemistry for p65 (RelA), the large subunit of NF-κB. IVD cells were seeded on chamber slides and were grouped into no treatment, IL-1β treatment (5 ng/mL) or IL-1β (5 ng/mL) + resveratrol treatment (50 μM). Treatment was performed for 5, 15, 30, 60, or 120 minutes or 18 hours before fixing cells for 10 minutes in ice-cold methanol. After washing, cells were blocked with 1% bovine serum albumin (BSA) and 0.1% Triton-X in PBS for 60 minutes, incubated with p65 antibody for 60 minutes, washed, incubated with the respective secondary fluorescence-labeled antibody for 45 minutes, embedded in Mowiol 4–88 and microscopically analyzed. The assay was performed on cells from two independent donors.

p65 and MAP Kinase (ERK, p38, JNK) Analysis

NF-κB (p65)

To further investigate the influence of resveratrol on the NF-κB pathway, IVD cells were grouped into no treatment, IL-1β treatment (5 ng/mL) or IL-1β (5 ng/mL) + resveratrol treatment (50 μM). After 60 minutes, nuclear extracts were prepared according to standard protocols. Protein content was measured by Bradford assay, nuclear extracts were fractioned by SDS-PAGE, proteins were transferred onto membranes and first incubated with a p65 antibody and then with the

appropriate secondary antibody before analyzing chemiluminescence. Poly (ADP-ribose) polymerase-1/2 (PARP-1/2) was used as the loading control. The assay was performed on samples from three independent experiments.

MAP Kinases (p38, ERK, JNK)

To investigate whether resveratrol acts on typical MAP kinases, IVD cells were grouped into no treatment, IL-1β treatment (5 ng/mL) or IL-1β (5 ng/mL) + resveratrol treatment (50 μM). After 15 minutes, whole cell extracts were prepared according to standard protocols. Whole cell extracts were used as stated earlier, but incubated with either unphosphorylated or phosphorylated antibodies recognizing p38, ERK (p42/44) or JNK before adding an HRP-labeled rabbit secondary antibody and analyzing chemiluminescence. Tubulin was used as a loading control. The assay was performed on samples from three independent experiments.

Electrophoretic Mobility Shift Assay for NF-κB

Effects of resveratrol on NF-κB activity were further confirmed by performing electrophoretic mobility shift assay (EMSA) for NF-κB on nuclear extracts from IVD cells, which were grouped into no treatment, IL-1β treatment (5 ng/mL) or IL-1β (5 ng/mL) + resveratrol treatment (50 μM) (at 60 minutes). By subjecting samples to electrophoretic mobility shift analysis, the binding of NF-κB to DNA can be visualized. Briefly, nuclear extracts were incubated with binding buffer for 10 minutes before adding biotin-labeled NF-κB probe, followed by incubation at 15°C for 30 minutes. Samples were electrophoretically separated on a 6% polyacrylamide gel, transferred onto a nylon membrane, and then fixed by UV cross-linking. Specificity of the complex was confirmed with a competition assay by addition of a non–biotin-labeled cold probe as recommended by the manufacturer (signal removed). The assay was performed on samples from two independent experiments.

In Vivo Animal Experiments on Pain Behavior

All animal experiments were carried out under the control of the Animal Care and Use Committee in accordance with local guidelines for the animal experiments and government law concerning the protection and control of animals.

Female Sprague-Dawley rats (n = 18, 200–250 g) (Japan SLC, Shizuoka, Japan) were anesthetized by intraperitoneal injection of 30 mg/kg sodium pentobarbital, placed in a prone position and surgical intervention was performed by use of a stereo operating microscope and microsurgical instruments. Briefly, an incision was made to the spinal midline, fascia and multifidus muscle were resected, and the left L5 nerve root and dorsal root ganglion (DRG) were exposed by L5-L6 facetectomy on the left side, with great care taken to avoid trauma to the tissue. Autologous NP was harvested from the tail and applied to the DRG in 12 animals. In addition, either resveratrol (0.1 mL of 50 μM solution in saline [NPR group]) or saline (0.1 mL [NPS group]) were injected into the underlayer of the epineurium just distal to the NP in 6 animals each before closing the incisions. In the Sham group (n = 6), the left L5 nerve root and DRG were exposed by L5-L6 facetectomy on the left side, but no other procedures were performed. All animals underwent behavioral testing at days 0 (baseline), 2, 7, 14, and 21 by evaluating the hind paw withdrawal response to von Frey hair stimulation of the plantar surface of the footpad. Briefly, von Frey filaments with a calibrated force between 1 and 29 g were sequentially applied twice to the paw surface. The determined withdrawal force was verified with a negative test of next lower filament as well as by confirming the initial response after a time lag of 5 minutes. Lower withdrawal thresholds are considered a sign of mechanical hypersensitivity, which is correlated to pain behavior in this animal model.

Statistical Analysis

All quantitative cell culture data were expressed as means and standard error of the mean (SEM). Statistical analysis was performed by Mann-Whitney *U* test (two-tailed) using the SPSS software. A significance level of $P < 0.05$ was considered statistically significant. For the animal study, behavioral data were also statistically evaluated by analysis of variance (ANOVA) with both nonparametric and multiple comparisons by Bonferroni correction test among the three treatment groups.

RESULTS

Cell Viability

As shown in Figure 1, resveratrol did not show any cytotoxic effect after 18 hours at concentrations ≤ 250 μM.

Effects of Resveratrol on the Gene Expression Level

As expected, prestimulation with IL-1β caused a strong upregulation of IL-1β, IL-6, IL-8, MMP1, MMP3, MMP13

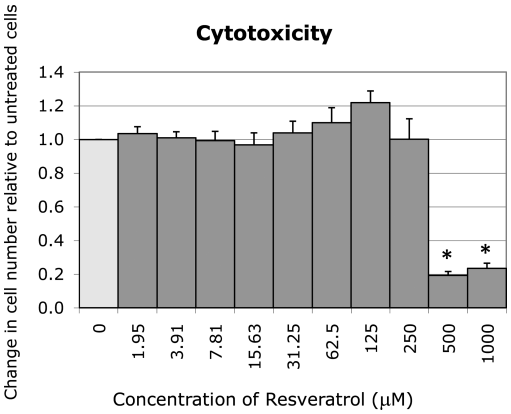

Figure 1. Resveratrol is not cytotoxic at moderate concentrations. Resveratrol had no cytotoxic effect at concentrations ≤ 250 μM, but was significantly cytotoxic at 500 μM ($P = 0.002$) and 1000 μM ($P = 0.003$) after 18 hours. Data were obtained by use of the MTT assay and is presented as Mean and SEM (n = 6). Asterisks indicate statistical significance ($P < 0.05$).

TABLE 4. Stimulation With IL-1β Induces an Inflammatory and Catabolic Response. Stimulation With 5 ng/mL Recombinant IL-1β Increased mRNA Levels of IL-1β, IL-6, Il-8, MMP1, MMP3, MMP13, and TLR2. This Table Summarizes Means, SEM, and *P* Values for all Genes of Interest. Data Were Obtained by Real-Time RT-PCR ($2^{-\Delta\Delta Ct}$ Method) (n = 6)

Gene	Mean (Fold Change)	SEM	*P*
Interleukin-1β (IL-1β)	8.66	3.02	0.003
Interleukin-6 (IL-6)	44.37	10.88	<0.001
Interleukin-8 (IL-8)	11.70	3.51	<0.001
Toll-like receptor 2 (TLR2)	3.44	0.78	0.003
Matrixmetalloproteinase-1 (MMP1)	39.40	11.11	<0.001
Matrixmetalloproteinase-3 (MMP3)	90.81	29.04	<0.001
Matrixmetalloproteinase-13 (MMP13)	13.28	2.19	<0.001

and TLR2 (Table 4). Resveratrol exhibited minor or no effects at the lower concentration (5 μM) (Figure 2). However, at the higher concentration (50 μM), resveratrol caused a significant inhibition of the IL-1β–mediated inflammatory and catabolic response. After setting IL-1β–induced gene expression to 100% for each gene, resveratrol was able to reduce expression of proinflammatory cytokines (IL-6 by 76%, IL-8 by 55%), matrix metalloproteinases (MMP1 by 51%, MMP3 by 48%, MMP13 by 45%) and TLR2 by 18% (Figure 2).

Effects of Resveratrol on the Protein Expression Level

The anti-inflammatory and anticatabolic effect of the highest concentration of resveratrol that was observed on the mRNA level could be confirmed on the protein level for IL-6, IL-8, MMP1, MMP3, and MMP13 (Figure 3). The general observation was that treatment with IL-1β–induced protein levels of all candidates compared with untreated control cells (lanes 1 and 2), but that 50 μM resveratrol was able to effectively reduce IL-1β–induced levels in all donors (lane 3). For the investigated matrix degrading enzymes, it should be noted that both, proform and active form, were reduced with resveratrol treatment.

Effects of Resveratrol on NF-κB Activity

Treatment of IL-1β prestimulated cells with 50 μM resveratrol did not prevent or reverse nuclear translocation of the NF-κB subunit p65 at any time point. In resveratrol treated cells, the specific p65 antibody detected p65 in the nucleus (just as in the cells treated with IL-1β only, which serves as a positive control

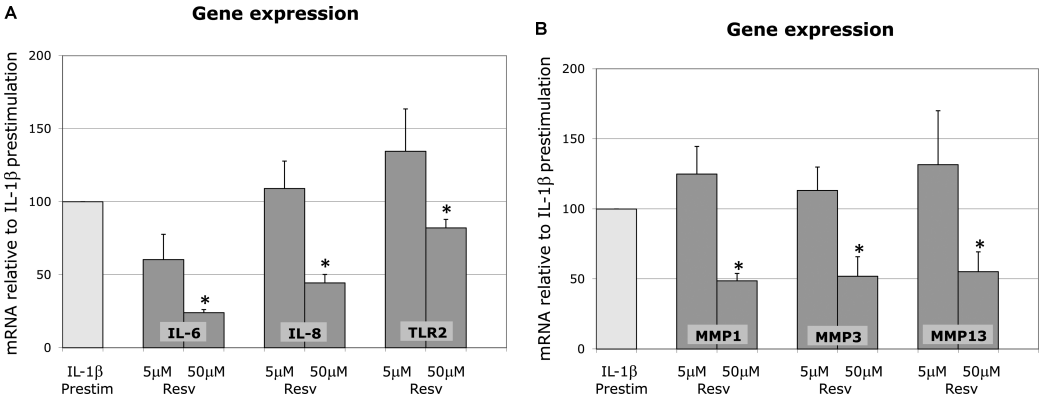

Figure 2. Resveratrol exhibits an anti-inflammatory and anticatabolic effect on the mRNA level. After 18 hours, 50 μM resveratrol reduced mRNA levels of proinflammatory cytokines IL-6 ($P < 0.0001$) and IL-8 ($P < 0.0001$) (**A**), Toll-like receptor TLR2 ($P = 0.003$) (**A**), and matrix-degrading enzymes MMP1 ($P < 0.0001$) (**B**), MMP3 ($P = 0.003$) (**B**) and MMP13 ($P = 0.046$) (**B**) compared with IL-1β–prestimulated cells, but had no effect on mRNA levels of IL-1β (not shown). Data were obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 6). Asterisks indicate statistical significance ($P < 0.05$).

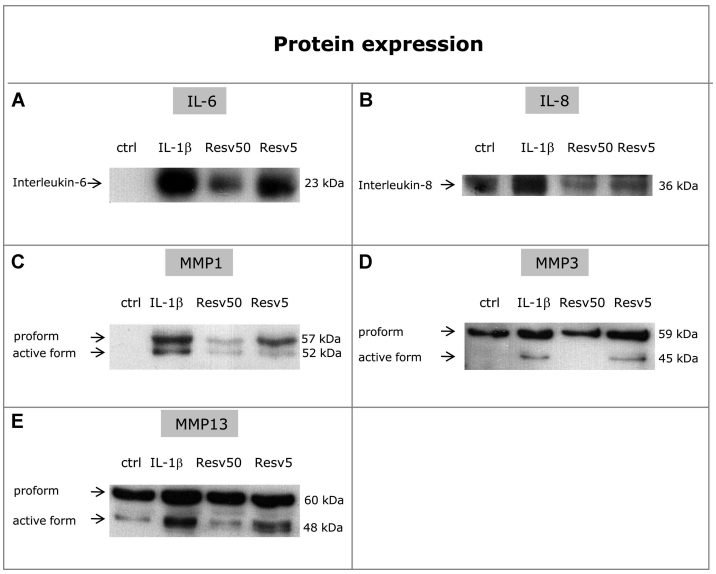

Figure 3. Resveratrol exhibits an anti-inflammatory and anticatabolic effect on the protein level. After 18 hours, 50 μM resveratrol reduced protein levels of IL-6 (**A**), IL-8 (**B**), MMP1 (**C**), MMP3 (**D**), and MMP13 (**E**), compared with IL-1β–prestimulated cells. Data were obtained by immunoblotting of protein samples extracted from the conditioned medium (n = 3) and one representative sample is shown.

of NF-κB induction), while untreated cells show cytoplasmic location of p65 (which is indicative of nonactive NF-κB) (Figure 4A). This finding was confirmed by p65 immunoblotting of nuclear extracts (Figure 4B): Western blots clearly show that the p65 band in the nuclear extract of resveratrol treated samples is not reduced compared with IL-1β–stimulated samples, while untreated cells show a much smaller amount of target protein. EMSA experiments, revealed no decrease or elimination of the NF-κB DNA-binding activity in resveratrol treated cells compared with IL-1β prestimulated cells (Figure 4C), confirming results obtained by Western blot and immunocytochemistry. Specificity of the EMSA assay was confirmed by using a cold probe in one lane (negative control), which has no visible band.

Effects of Resveratrol on SIRT1 Activity

To test whether resveratrol exhibits its effects by activating SIRT1, resveratrol-treated cells were cotreated with sirtinol, a SIRT1 inhibitor. Cotreatment of resveratrol and sirtinol did not overall abolish inhibition of gene expression observed by resveratrol treatment alone (Figure 5). Only for MMP1, cotreatment of resveratrol and sirtinol resulted in an increase in gene expression compared with resveratrol treatment alone (5 μM: $P = 0.019$; 10 μM: $P = 0.013$). In contrast, MMP3 and MMP13 expression was even further reduced upon sirtinol cotreatment compared with resveratrol treatment alone (MMP13 at 5 μM: $P = 0.003$; MMP13 at 10 μM: $P = 0.006$; MMP3 at 10 μM: $P < 0.0001$).

Effects of Resveratrol on MAP Kinase Activity

Involvement of typical MAP kinases in the effects observed by resveratrol treatment was investigated by detecting levels of phosphorylated and unphosporylated p38, ERK and JNK in whole-cell extracts by immunoblotting (Figure 6). Results demonstrate that IL-1β treatment increased levels of phosporylated p38, ERK, and JNK after 15 minutes, which is indicative of activation of these three MAP kinases. However, treatment with 50 μM resveratrol did not influence activity of any of the three MAP kinases. Levels of unphosporylated p38, ERK, and JNK were similar in all groups.

Effects of Resveratrol on Pain Behavior in an Animal Model

Animal behavior in response to nonnoxious mechanical stimulation with von Frey filaments was compared with the sham group, which (as expected) showed a rather stable mechanical withdrawal. Withdrawal thresholds in the control group were overall close to the preoperative baseline over the entire course of the experiment, indicating that the surgical intervention alone did not cause any change in pain behavior. The additional two groups (treatment with NP tissue + saline = NPS, treatment with NP tissue + resveratrol = NPR) were compared with the sham group for all time points. Results indicate that in the NPS group, the mechanical withdrawal thresholds were significantly decreased for each time point compared with the baseline values of the sham group up to day 14, indicating that pain was evoked by application of NP tissue to the DRG. In the NPR group that was cotreated with resveratrol, animal behavior was very similar to the sham group. Thresholds in the NPR group were significantly higher than in the NPS group on day 2 ($P < 0.0001$), day 7 ($P = 0.037$) and day 14 ($P = 0.0044$). However,

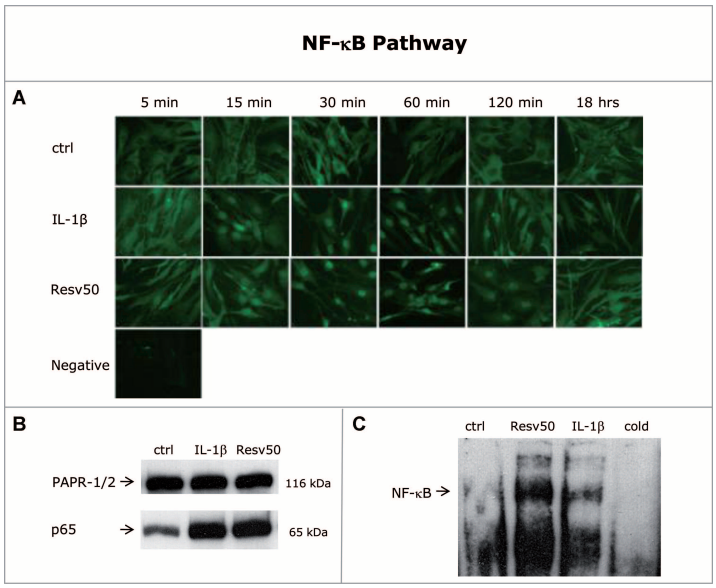

Figure 4. Resveratrol does not seem to inhibit NF-κB induction. Part **A** demonstrates that untreated control cells show cytoplasmic localization of p65 (indicative of inactive NF-κB), while IL-1β-alone and IL-1β+resveratrol treated samples show nuclear localization of p65 (indicative of NF-κB induction). Data were obtained by immunocytochemistry for p65 (n = 2) and one representative sample is shown. Part **B** shows increased levels of p65 in nuclear extracts upon stimulation with IL-1β (indicative of NF-κB induction) compared with untreated control cells, but this effects was not abolished by resveratrol, therefore confirming data shown in Part **A**. Data were obtained by p65 immunoblotting of nuclear extracts (n = 3) and one representative sample is shown. PARP-1/2 is used as a loading control. In Part **C**, the presence of NF-κB DNA binding can be observed in both, IL-1β-alone and IL-1β+ resveratrol-treated samples (indicative of NF-κB induction), but not in untreated control samples (indicative of inactive NF-κB). Treatment with a provided cold probe abolished the signal, thus confirming specificity of the assay. Data were obtained by p65 electrophoretic mobility shift assay (EMSA) of nuclear extracts (n = 2) and one representative sample is shown.

at day 21, there was no significant difference anymore between the thresholds of the NPR and NPS (Figure 7).

DISCUSSION

Anti-Inflammatory and Anticatabolic Potential of Resveratrol

Results from our cell culture experiments provide clear evidence that resveratrol can effectively reduce mRNA levels of major proinflammatory cytokines (IL-6, IL-8), TLR2, and matrix degrading enzymes (MMP1, MMP3, MMP13), which have previously been shown to be involved in disc degeneration and pain induction. This effect could be confirmed on the protein expression level for IL-6, IL-8, MMP1, MMP3, and MMP13. Very few donors seemed to be unresponsive to resveratrol treatment, indicating that genetic differences may possibly influence treatment outcomes. Data from these patients were excluded from the analysis as these cells simultaneously responded in an excessive manner to IL-1β treatment. No demographic differences of these patients could be identified. The observed inhibition of the expression of certain genes is similar to what was observed in chondrocytes.[20,22] However, resveratrol also regulated expression of IL-1β in chondrocytes,[23] whereas IL-1β was not regulated in our experiments on human IVD cells.

In a study performed by Li *et al*[24], resveratrol has additionally been shown to increase proteoglycan synthesis and to rescue IL-1β–induced proteoglycan loss in bovine IVD cells, therefore providing further evidence that resveratrol may be an innovative treatment of NP-mediated back and leg pain. Li *et al*[24] cultured their bovine disc cells in both two dimensions and alginate, but we concentrated on a two- dimensional culture approach. Although this is a limitation of the present study as phenotypic shifts during monolayer culture may affect the results, this approach enabled us to perform the subsequent detailed signal transduction analysis (with the goal to further elucidate the molecular mechanisms underlying the anti-inflammatory and anticatabolic effects of resveratrol) in the same system.

Pathway Analysis (NF-κB)

The activation of the transcription factor NF-κB leads to an upregulation of proinflammatory cytokines and matrix degrading enzymes. We therefore investigated whether resveratrol exhibits its effects via inhibition of NF-κB. In addition, we investigated

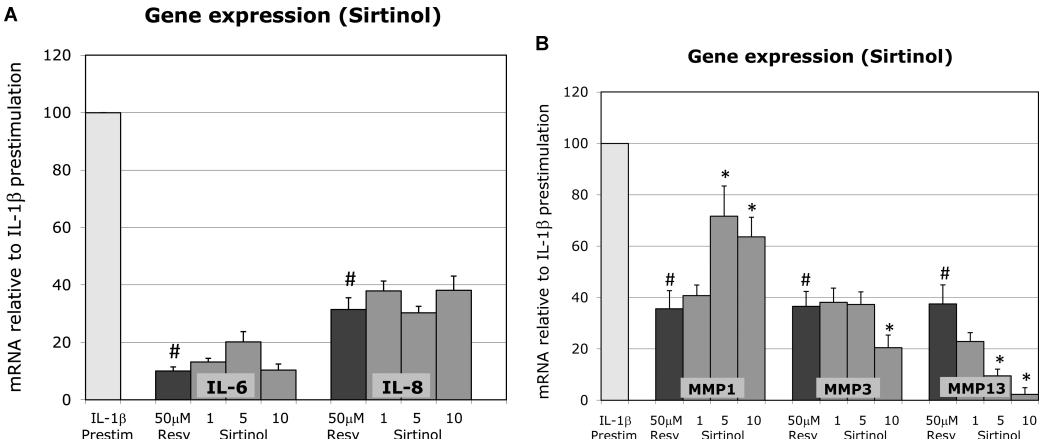

Figure 5. Resveratrol does not seem to act as a SIRT1-activator. Cotreatment of IL-β prestimulated cells with resveratrol (50 μM) and the SIRT1 inhibitor sirtinol (1, 5, or 10 μM) did not abolish the effects observed by resveratrol treatment alone (reduced mRNA expression of IL-6, IL-8, MMP1, MMP3, MMP13) after 18 hours, with the exception of a partial reversal of MMP1 expression at 5 μM ($P = 0.019$) and 10 μM ($P = 0.013$). Expression of MMP3 and MMP13 was further reduced on treatment with 5 μM sirtinol (MMP13: $P = 0.003$) and 10 μM sirtinol (MMP3: $P = 0.006$; MMP13: $P < 0.0001$). Part **A** shows proinflammatory cytokines, Part **B** shows MMPs. Data were obtained by real-time RT-PCR ($2^{-\Delta\Delta Ct}$ method) and is presented as Mean and SEM (n = 6). Rhombi indicate statistical significance between IL-1β prestimulation and resveratrol treatment; asterisks indicate statistical significance between resveratrol treatment and resveratrol + sirtinol treatment ($P < 0.05$).

regulation of TLR2, as activation of TLR2 is known to trigger several crucial intracellular signaling responses including activation of the transcription factor NF-κB. Although resveratrol was shown to inhibit expression of certain TLRs for example in HEK293 and RAW264.7 cells,[25,26] only a minor reduction of TLR2 was observed in IVD cells. We also found that resveratrol

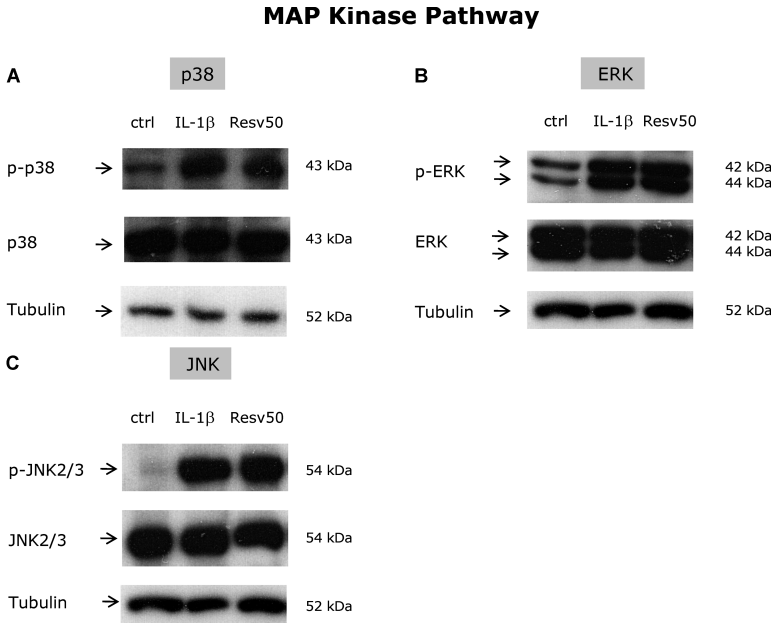

Figure 6. Resveratrol does not seem to act on the MAP Kinase pathway. Although treatment with IL-1β induced levels of phosporylated p38, ERK, and JNK compared with untreated control cells, treatment with 50 μM resveratrol was not able to attenuate activation of any of the members of the MAP kinase family (6A: p-p38, 6B: p-ERK, 6C: p-JNK). Levels of unphosporylated p38 (**A**), ERK (**B**), and JNK (**C**) are similar in all groups. Data were obtained by immunoblotting of whole-cell extracts (n = 3) and one representative sample is shown. Tubulin is used as the loading control in all blots.

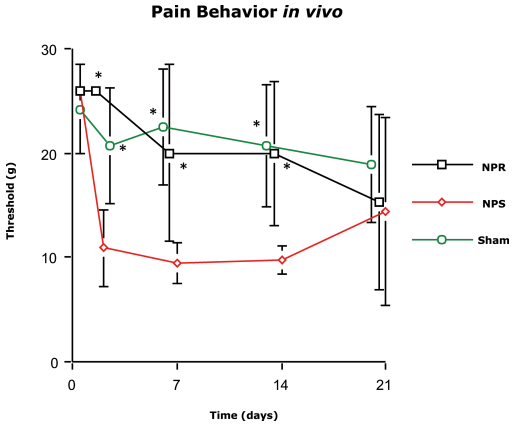

Figure 7. Resveratrol reduces pain behavior *in vivo*. This figure shows results of the *in vivo* testing of resveratrol, using a rodent pain model. Compared with sham animals, application of nucleus pulposus tissue (+ saline) (NPS group) reduced mechanical sensitivity thresholds primarily up to day 14. If coapplication of nucleus pulposus tissue and resveratrol was performed at the DRG (NPR group), thresholds were significantly higher than in the NPS group at days 2 ($P < 0.0001$), 7 ($P = 0.037$), and 14 ($P = 0.0044$) and very similar to the sham group (indicative of pain reduction by resveratrol treatment). Data were obtained by von Frey filament testing and is presented as Mean and SD (n = 6). Asterisks indicate statistical significance ($P < 0.05$).

was not able to attenuate the nuclear translocation of p65 as shown by Western blot on nuclear extracts and immunocytochemistry and also did not decrease the NF-κB DNA-binding activity as shown by EMSA. Our study indicates that resveratrol does not seem to act on the NF-κB pathway in human IVD cells, although this has been observed in multiple other cell types[19,27–30].

Pathway Analysis (SIRT1)

As resveratrol is considered to be a potent SIRT1 activator[31] and as SIRT1 is known to inhibit the transcriptional activity of NF-κB, resveratrol is thought to exhibit its anti-inflammatory effect via this pathway.[32–36] We performed experiments to test for involvement of the SIRT1 pathway in resveratrol-treated human IVD cells by costimulating cells with resveratrol and the SIRT1 inhibitor sirtinol. Sirtinol was not able to attenuate the anti-inflammatory and anticatabolic effect of resveratrol in IVD cells (except partially for the gene MMP1), thus indicating that resveratrol does not seem to exhibit its effect via the SIRT1 pathway. Instead of attenuating the anticatabolic effect of resveratrol, treatment with the SIRT1 inhibitor resulted in even further reduced mRNA levels of MMP3 and MMP13. A most recent study by Rothgiesser *et al*[37] indicates that, depending on the lysine position in p65, acetylation can cause an increase or a decrease of gene expression.

Pathway Analysis (MAP Kinases)

Although resveratrol has been described as an inhibitor of the activity of one or several members of the MAP kinase family in multiple studies on various cell types (p38,[38–40] ERK,[38,40,41] JNK[38,42]), we could not confirm this mode of action in human IVD cells.

Animal Study

We used an *in vivo* animal model in which NP-mediated pain is simulated by the application of NP tissue to the DRG, thus representing typical radiculopathic pain. Similar to previous studies,[43–46] NP tissue induced behavior related to nociceptive pain in our animal experiments. Resveratrol treatment was able to prevent this threshold reduction and thus the pain-related behavior to a certain degree for 14 days. However, there was no significant difference of the thresholds between resveratrol and saline after NP treatment after 21 days. A more pronounced and long-lasting effect of resveratrol may be obtained by varying its concentration or the type of administration. As induction of nociceptive pain in this model is thought to be due to the release of, for example, TNF-α, interleukins, and serotonin,[5,8,44] we speculate that resveratrol may possibly reduce pain behavior *in vivo* by reducing or inhibiting cytokines that are released from the NP tissue, similar to the mechanism observed in our *in vitro* cell culture study. Despite these promising findings, further experimental studies, for example, the use of controlled drug release systems or a large animal model are needed before resveratrol can be considered for clinical use. One limitation of this study is that the *in vivo* animal model did not ideally match the stepwise approach of our *in vitro* model. *In vivo*, IL-1β and resveratrol were applied simultaneously, whereas a stepwise approach with IL-1β pretreatment was performed in the *in vitro* part. Although pretreatment better simulates the disease process, this would lead to a second surgery, thus increasing stress and pain for the animals and the risk of surgery-related issues. As the investigation of pain behavior in rodents is challenging and prone to any interference, we chose to perform the less invasive (simultaneous) approach.

➢ Key Points

- ❑ As production and release of proinflammatory cytokines seem to play a pivotal role in nucleus pulposus (NP)–mediated pain (discogenic pain, radiculopathy), anti-inflammatory substances might be used to more specifically treat affected patients.
- ❑ Resveratrol, a polyphenol found in red wine, was able to reduce expression of relevant proinflammatory cytokines and matrix degrading enzymes on the gene and protein expression level in human intervertebral disc cells *in vitro*.
- ❑ Although there is clear indication in the literature that resveratrol inhibits toll-like receptor 2 (TLR2) and hence downstream activation of NF-κB and activates SIRT1, only a minor change in TLR2 expression and no effect on NF-κB and SIRT1 could be detected with the methods used in this study. Resveratrol also did not seem to exhibit its effects via the MAP kinases, p 38, ERK, or JNK.
- ❑ The analgetic potential of resveratrol to reduce NP-mediated pain could be demonstrated in an *in vivo* animal model of painful radiculopathy.

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Basic Science

Bupivacaine—the deadly friend of intervertebral disc cells?

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Abstract

BACKGROUND CONTEXT: Bupivacaine is commonly used as an adjunct during provocative discography and is administered intradiscally in patients with discogenic back pain. Recent studies demonstrated that bupivacaine is cytotoxic for articular chondrocytes in vitro at clinically used concentrations (0.25%–0.5%).

PURPOSE: To analyze a concentration-dependent effect of bupivacaine on cell viability and gene expression of human intervertebral disc (IVD) cells in an in vitro model.

STUDY DESIGN: In vitro cell culture study.

PATIENT SAMPLE: Disc cells were isolated from human disc biopsies from 11 patients undergoing surgery because of degenerative disc disease or disc herniation.

OUTCOME MEASURES: Cell viability and gene expression after exposure to bupivacaine.

METHODS: Human IVD cells were treated with different concentrations of bupivacaine for 2 (n=5) or 18 hours (n=5) and analyzed for cell viability and proliferation (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay). Additionally, cells were prestimulated with interleukin-1 beta (IL-1β) (5 ng/mL) to increase the levels of proinflammatory cytokines and matrix-degrading enzymes and thereafter treated with 0.75 mmol bupivacaine (as determined in the cell viability test) for 2 (n=5) or 18 hours (n=5). Prestimulated cells with or without bupivacaine treatment were analyzed for gene expression of IL-1β, IL-6, IL-8, tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), matrix metalloproteinase-3 (MMP3), MMP9, MMP13, and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) using real-time reverse transcription-polymerase chain reaction. Statistical analysis was performed by using the Mann-Whitney *U* test with a significance level of $p<.05$.

RESULTS: After 18 hours, bupivacaine exhibited either a cytotoxic or a proliferative effect on human IVD cells, depending on the concentration. Similar but lower effects could be observed already after 2 hours. With a concentration of 0.75 mmol (proliferative effect), bupivacaine significantly decreased messenger RNA levels of TNF-α, COX-2, MMP13, and ADAMTS4 after 18 hours. In contrast, expression of IL-6, IL-8, and MMP9 did not differ; expression of IL-1β and MMP3 was stimulated with 0.75 mmol. After 2 hours, we observed a reduction in the expression of COX-2, MMP3, MMP13, and ADAMTS4, without any effect regarding IL-1β.

CONCLUSIONS: Application of bupivacaine in clinically relevant concentrations was toxic for IVD cells in vitro. A low concentration stimulated cell proliferation and reduced gene expression of certain matrix-degrading enzymes and proinflammatory cytokines. If these results can be corroborated in tissue explant models or animal studies, caution regarding provocative discography with bupivacaine is prompted.

Keywords: Human intervertebral disc cells; Bupivacaine; Cytotoxicity; Gene expression; Provocative discography

FDA device/drug status: not applicable.

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Introduction

Spinal injections are frequently used for the diagnosis and treatment of patients with low back and leg pain. A variety of different drugs such as local anesthetics or corticosteroids are used, and the choice of technique depends on whether the aim is to eliminate or to diagnose the spinal pain [1].

Various reports on the intradiscal injection of bupivacaine with the aim to eliminate or reduce pain can be found in the literature, with different success rates in various studies [2–4]. In patients with one-level internal disc disruption or nonsequestered nuclear prolapse, intradiscal injection of 0.5% bupivacaine was previously tested, but only 9% of the treated patients showed clinical improvement [2]. In contrast, in another study including patients with chronic low back pain, intradiscal administration of 0.5% bupivacaine resulted in immediate improvement in 83% of the patients. This improvement was maintained for 2 weeks in 67% of patients but only in 17% of patients after 1 month [3]. Intradiscal injection of 50% dextrose and 0.25% bupivacaine in patients with severe degenerative disc disease resulted in sustained improvement in 43.4% of patients, although it is unclear which of the two components was primarily responsible for this clinical effect [4]. The pain relieving mechanism of local anesthetics such as bupivacaine can be twofold: It can inhibit sensitization of nerve endings, which is thought to be the main mechanism underlying pain relief. This is based on the inhibition of certain voltage-gated ion channels (sodium and potassium), therefore reducing the action potential in the neural cell [5,6]. Latest studies indicate that it may also reduce production of proinflammatory cytokines [7–10]. It could, for example, be demonstrated that bupivacaine leads to a cytokine reduction in macrophages (interleukin-1 beta [IL-1β], IL-6, tumor necrosis factor-α [TNF-α]) [7] and rat fibroblasts (IL-2, IL-4, and their receptors) [10] and to an inhibition of carrageenan-evoked systemic cytokine production in rats [9]. Carrageenans are a family of linear sulfated polysaccharides, which are extracted from red seaweeds and have an ability to form gels. A group of local anesthetics (lidocaine, bupivacaine, and amethocaine) were additionally shown to inhibit secretion of IL-8 and IL-1β in HT-29 and Caco-2 cell lines (intestinal epithelial cells) and partially in freshly isolated epithelial cells in a concentration-dependent manner [8].

For diagnosis of discogenic back pain, provocative discography is commonly used, in which spinal pain can be provoked by the injection of contrast medium. The mechanism of pain provocation during discography is largely unknown, but it is hypothesized that pathological metabolites are extruded from the disc and irritate nerve fibers in the outer annulus fibrosus, thus causing pain [11]. After contrast medium injection, local anesthetics such as bupivacaine at a concentration of 0.25% or 0.5% are commonly used to reduce the pain [12,13]. Provocative discography remains a topic of debate. Recently, Carragee et al. [14] demonstrated that provocative discography seems to result in an accelerated disc degeneration/herniation and a loss in disc height/signal after 10 years. The procedure was performed using a 22-gauge to 25-gauge needle, but no mention is made concerning the use of a contrast medium and/or a local anesthetic. The negative effects of discography could be based on tissue rupture at the injection side because of the needle, on the contrast medium that is used to provoke the pain, or on the local anesthetic that is commonly injected at the end of the procedure.

Negative effects of bupivacaine have been reported, especially with regard to its toxicity. Recent in vitro studies on articular chondrocytes elucidated that bupivacaine was cytotoxic at the clinically applied concentrations [15–17]. It could be demonstrated by Chu et al. [15] that exposing human articular chondrocytes for 15 minutes to 0.25% bupivacaine resulted in 41% reduced viability, whereas 0.125% seemed to have no cytotoxic effect. Cytotoxicity of 0.25% bupivacaine on chondrocytes could be confirmed under in vivo conditions as well, using continuous infusion into the shoulder of rabbits [18]. So far, the response of intervertebral disc (IVD) cells to bupivacaine treatment is not well explored. Only one study published in October 2009 exists so far, which indicates that bupivacaine may possess strong cytotoxicity for disc cells as well, similar to articular chondrocytes [19].

The objective of this study, therefore, was to analyze the potential concentration-dependent cytotoxic effect of different concentrations of bupivacaine on human IVD cells. An additional objective was to determine the effects of bupivacaine on proinflammatory cytokines and matrix-degrading enzymes.

Methods

Cell isolation and culture

Intervertebral disc tissue was obtained from a total of 11 patients (Table 1). All patients underwent a discectomy for disc herniation or interbody fusion for degenerative disc disease. The material was obtained from a spinal service of a University Hospital.

In most samples, a differentiation of the nucleus pulposus and annulus fibrosus tissue was not possible because of degeneration. Therefore, a mixed cell population (nuclear chondrocytes and annular fibroblasts) was used. Cells were isolated by overnight digestion with 0.2% collagenase NB4 (Serva, Switzerland) and 0.3% dispase II (Roche Diagnostics, Switzerland) in phosphate-buffered saline (37°C, 5% carbon dioxide). Digested tissue was filtered (70 μm cell strainer, BD Bioscience, Switzerland) and washed with Dulbecco's Modified Eagle's Medium: Ham's Nutrient Mixture F-12 (DMEM/F12) medium (Sigma, Switzerland). Cells were expanded up to two passages in a monolayer using DMEM/F12 medium, containing 10% fetal calf serum,

Table 1
Patient data

No.	Sex	Age (y)	Pathology	Grade	Disc level
1	M	29	Sequestration	III	L5/S1
2	F	66	Protrusion	IV	L4/L5
3	M	61	Sequestration	IV	L3/L4
4	F	50	Protrusion	II	L3/L4
5	F	29	Sequestration	IV	L4/L5
6	M	55	Sequestration	IV	L4/L5
7	M	41	Unknown	Unknown	Unknown
8	M	48	Extrusion	IV	L4/L5
9	M	72	Sequestration	III	L3/L4
10	M	34	Sequestration	III	L4/L5
11	M	36	Extrusion	IV	L4/L5

M, male; F, female; Grade, Pfirrmann grade for degeneration.

Cells from several donors were used for more than one method or time point.

Table 2
Primers/probes used for real-time reverse transcription-polymerase chain reaction

Gene	Primer sequence number	Base pairs
TATA-box binding protein	HS 00427620_m1	91
Interleukin-1β	HS 00174097_m1	94
Interleukin-6	HS 00174131_m1	95
Interleukin-8	HS 00174103_m1	101
Tumor necrosis factor-α	HS 00174128_m1	80
Cyclooxgenase-2	HS 00153133_m1	75
Matrix metalloproteinase-3	HS 00968308_m1	98
Matrix metalloproteinase-9	HS 00957555_m1	79
Matrix metalloproteinase-13	HS 00233992_m1	91
Aggrecanase-1 (ADAMTS4)	HS 00943031_g1	91

ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs.

penicillin (50 units/mL), streptomycin (50 μg/mL), and ampicillin (125 ng/mL) (Invitrogen, Switzerland) with medium changes once to twice a week.

Informed consent was obtained from all tissue donors before the procedure according to the requirement of the local institutional review board.

Cytotoxicity

Bupivacaine was dissolved in water to obtain a concentration of 150 mmol (stock), autoclaved, and diluted in DMEM/F12 medium containing 10% fetal calf serum, penicillin (50 units/mL), streptomycin (50 μg/mL), and ampicillin (125 ng/mL) to obtain concentrations of 0.1, 0.5, 1, 5, 7.5 (0.25%), and 15 mmol (0.5%).

Cells were seeded in 24-well plates with exactly the same cell density for all samples of one donor (approximately 50,000 cells/well for each donor) and expanded for a few days before adding 1,000 μL of bupivacaine medium in triplicates for each donor (of every concentration mentioned above). After 2 (n=5) and 18 hours (n=5), cells were analyzed for cell proliferation and viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay: A fresh sterile solution of MTT (Sigma, Switzerland) with a concentration of 0.5 mg/mL in the DMEM/F12 medium was prepared, 500 μL was added to each well and incubated for 3 hours at 37°C. MTT was discarded, cells were lysed with dimethyl sulfoxide for 10 minutes at 37°C, and absorbance was measured at 565 nm. Absorbance of bupivacaine-treated cells was calculated relative to absorbance of untreated control cells, which is set to 100%.

Gene expression

Expanded cells were maintained under serum-free conditions for 2 hours. Thereafter, 5 ng/mL of IL-1β (Peprotech, Switzerland) was added to all but one sample of each donor to induce an inflammatory response and to increase the levels of matrix-degrading enzymes, representing the putative situation in painful degenerated IVDs. Two hours later, bupivacaine stock was added to one pretreated sample per donor to obtain a concentration of 0.75 mmol. After 2 (n=5) or 18 hours (n=5), cells from all three conditions (untreated control cells, IL-1β prestimulation, IL-1β prestimulation+ 0.75 mmol) were harvested by trypsin treatment, and total RNA was isolated using the GenElute Mammalian total RNA Miniprep Kit (Sigma, Switzerland) according to the manufacturer's instructions. Complementary DNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Switzerland), and gene expression of IL-1β, IL-6, IL-8, TNF-α, cyclooxygenase-2 (COX-2), matrix metalloproteinase-3 (MMP3), MMP9, MMP13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS4), and TATA-box binding protein (a housekeeping gene: shown to be not regulated on treatment) was analyzed. Human specific probes and primers (Applied Biosystems, Switzerland, see Table 2), TaqMan real-time RT-PCR Mix (Applied Biosystems, Switzerland), and 10 ng of complementary DNA was mixed and measured in duplicates using the RG-3000A PCR machine (Corbett Research [now Qiagen], Switzerland). The comparative Ct method was used to calculate changes in gene expression of each measurement, first normalized versus the expression of TATA-box binding protein (ΔCt) and thereafter versus the untreated control or versus IL-1β (ΔΔCt). To calculate the change as a result of IL-1β stimulation, the ΔΔCt value was calculated between IL-1β–treated cells and control cells. To calculate the change as a result of bupivacaine treatment, the ΔΔCt value was calculated between bupivacaine-treated cells and IL-1β–treated cells. Results are indicated as a fold change in messenger RNA (mRNA), relative to untreated controls or IL-1β–treated cells, which is set to 1% or 100%, respectively.

Statistical analysis

The Mann-Whitney *U* test was used for analyzing statistical significance. The significance level was set as 0.05, two-tailed. Results in all figures are indicated as mean± standard error of the mean.

Results

Cytotoxicity

For cytotoxicity, cells under untreated control conditions were set to 100%. Values lesser than 100% represent cell death caused by bupivacaine, and values greater than 100% represent stimulated proliferation as a result of the treatment.

After 2 hours

When applying bupivacaine doses of 5 mmol and higher, a statistically significant toxic effect could be observed, with severe cell death happening at the clinically used concentrations of 7.5 and 15 mmol (0.25% and 0.5%), respectively ($p<.0001$ for both). A very slight but significant stimulation could be observed at 1 mmol ($p<.0001$) (Fig. 1, Left).

After 18 hours

When applying bupivacaine doses of 5 mmol (0.1%) and higher, a statistically significant toxic effect could be observed ($p<.0001$ for all). A cytotoxic effect (approximately 50%) could be observed already at 3 mmol (data not shown in Fig. 1, Right). Concentrations of 5 mmol and higher, which are clinically used, impaired cell viability with the cell death ratio reaching almost 100%. When using concentrations between 0.1 and 1 mmol, a significant stimulation of cell proliferation up to 170% could be observed (0.1 mM, $p=.003$; 0.5 mM, $p<.0001$; 1 mM, $p=.003$).

Effect on gene expression

Prestimulation with IL-1β

Cells were prestimulated with IL-1β for 2 hours (which is commonly used to induce the expression of proinflammatory cytokines and matrix-degrading enzymes in vitro) before starting another incubation period for 2 or 18 hours. The effects of prestimulation with IL-1β are compared with untreated control cells at the same time point, whose expression is set to one.

After 2 hours. Prestimulation with IL-1β resulted in a significant increase in the mRNA levels of ADAMTS4, MMP13, and COX-2, IL-8 (all <50-fold and $p<.015$) and IL-1β, MMP3, IL-6, and TNF-α (all >50-fold and $p<.015$) (Fig. 2A, B).

After 18 hours. Prestimulation with IL-1β resulted in a significant increase in the mRNA levels of ADAMTS4, MMP9, (all <50-fold and $p<.02$) and MMP13, IL-6, IL-8, and MMP3 (all >50-fold and $p<.0001$). No statistically relevant effect could be observed for TNF-α ($p=.42$) because of high variation between the donors (Fig. 2C, D).

Effect on mRNA levels of proinflammatory cytokines and matrix-degrading enzymes

After prestimulation, cells were treated with bupivacaine with a concentration of 0.75 mmol, under which proliferation was stimulated (see Fig. 1, Left, Right), and mRNA levels of proinflammatory cytokines and matrix-degrading enzymes were measured. The mRNA levels of prestimulated cells were set to 100%, and changes after bupivacaine treatment were depicted accordingly: Values lesser than 100% represent a decrease and values greater than 100% represent a stimulation of gene expression, as shown in Fig. 3.

After 2 hours. Compared with IL-1β prestimulation (100%), bupivacaine caused a significant reduction of most of the measured matrix-degrading enzymes (MMP3 to 60% with $p<.0001$, MMP13 to 57% with $p<.0001$, and ADAMTS4 to 62% with $p=.02$) except for MMP9, which was not regulated (Fig. 3A). Bupivacaine treatment also resulted in decreased COX-2 expression (to 56% with $p=.001$), whereas the remaining proinflammatory cytokines TNF-α, IL-1β, and

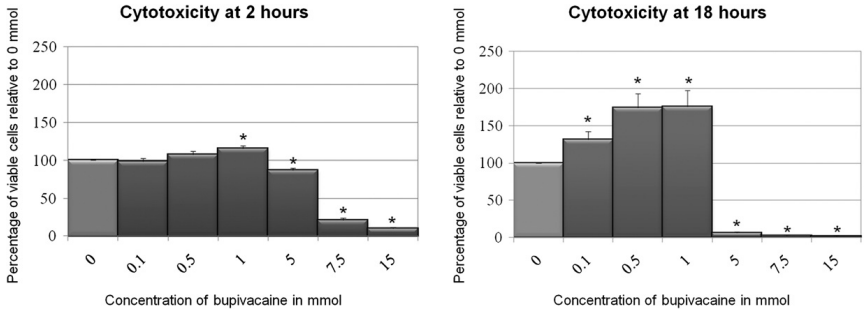

Fig. 1. Cytotoxicity of different concentrations of bupivacaine after (Left) 2 hours or (Right) 18 hours of treatment, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and calculated relative to untreated control conditions (100%). Data are indicated as mean±standard error of the mean. *$p<.05$ compared with untreated controls with $n=5$ in each group.

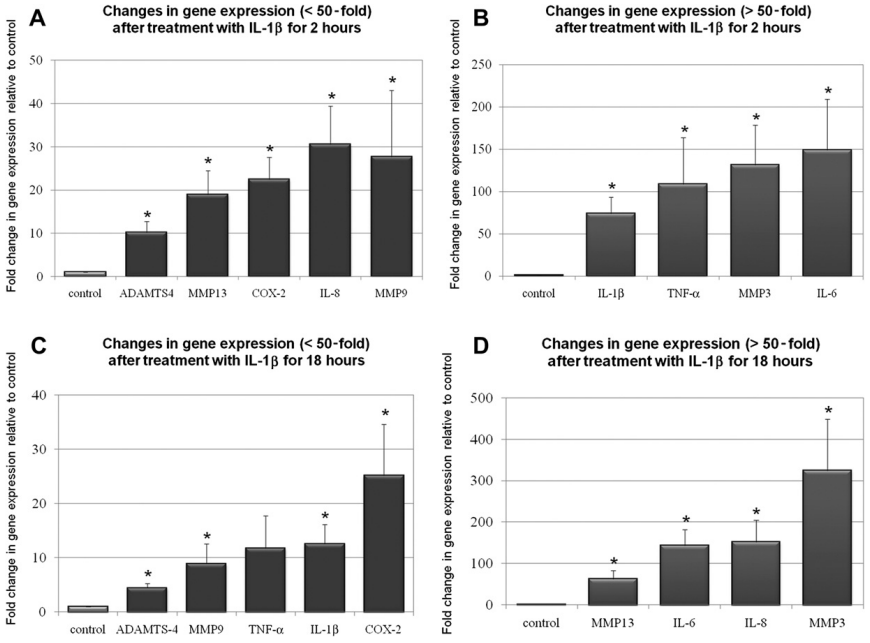

Fig. 2. Effects after IL-1β prestimulation. Gene expression after prestimulation with IL-1β was measured using real-time reverse transcription polymerase chain reaction for the (A, B) 2-hour group and (C, D) 18-hour group and compared with untreated control conditions (set to one). A, C show genes that are regulated less than 50-fold, and B, D show genes that are regulated more than 50-fold. Data are indicated as mean±standard error of the mean. *p<.05 compared with untreated controls with n=5 in each group. ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs; MMP13, matrix metalloproteinase-13; COX-2, cyclooxgenase-2; IL-8, interleukin-8; MMP9, matrix metalloproteinase-9; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; MMP3, matrix metalloproteinase-3; IL-6, interleukin-6.

IL-8 were not influenced; IL-6 was increased, but only slightly (130%), and this effect was not significant (p=.106) (Fig. 3B).

After 18 hours. Bupivacaine caused a significant decrease of MMP13 (to 25% with p<.0001) and ADAMTS4 (to 37% with p<.0001) compared with IL-1β prestimulation (100%). However, a minor increase of MMP3 could be observed as well (to 154% with p=.015) (Fig. 3C), and no statistically relevant effect was observed for MMP9. Bupivacaine treatment also resulted in a significant reduction of COX-2 (to 46% with p=.015) and TNF-α (to 55% with p=.015) compared with prestimulation, whereas no effect was seen for IL-6 and IL-8. On the other hand, bupivacaine treatment resulted in fourfold increased mRNA levels of IL-1β (to 418% with p<.0001) (Fig. 3D).

Discussion

Results from this study show that the local anesthetic bupivacaine, which is often used as an adjunct during provocative discography, strongly influences the viability and gene expression of human IVD cells in a concentration-dependent manner.

Cell viability

Cell viability measurement using the MTT assay clearly demonstrated that in vitro, concentrations typically used in a clinical setting (0.5%=15 mmol and 0.25%=7.5 mmol) resulted in severe cell death after both 2 and 18 hours of treatment. Although some crystallization occurred during incubation at the highest concentration (probably because of chemical incompatibility with the medium [20]), other concentrations also resulted in severe inhibition of cell viability, without showing any crystallization in this in vitro experiment. To further support our findings, MTT data were confirmed by two alternative cytotoxicity measurement methods for selected concentrations (0.75 mmol=nontoxic and 3 mmol=toxic) (picogreen assay and trypan blue; data not shown). The observed cytotoxicity (that was comparable in all measurements) is similar to that of a recently published article that demonstrated severe cell death of human and rabbit IVD cells caused by 0.25% or 0.5% bupivacaine

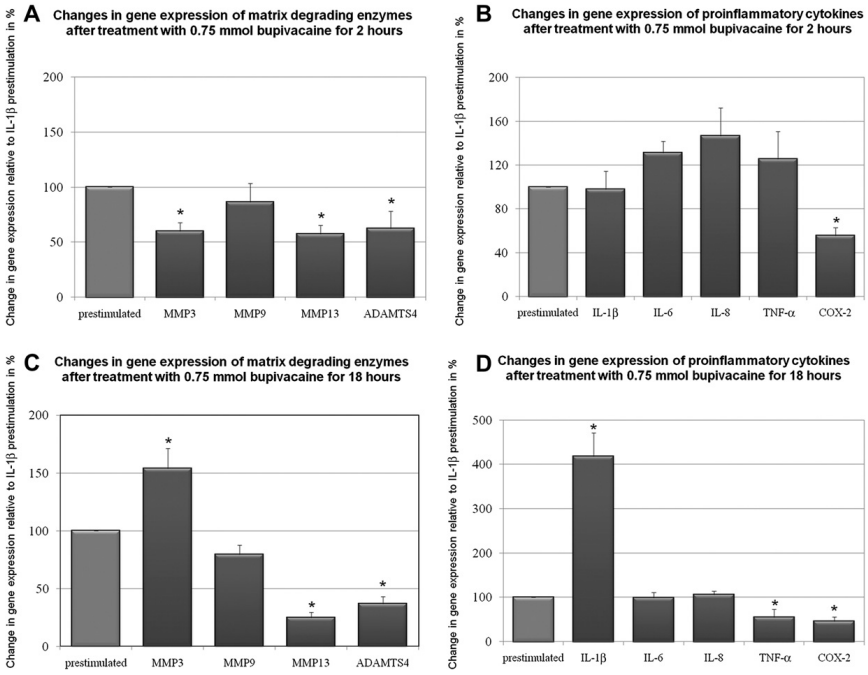

Fig. 3. Effects of bupivacaine treatment. Gene expression after treatment with bupivacaine (0.75 mmol) was measured using real-time reverse transcription polymerase chain reaction after (A, B) 2 hours and (C, D) 18 hours and compared with IL-1β–pretreated conditions (set to 100%). A, C show proinflammatory cytokines, and B, D show matrix-degrading enzymes. Data are indicated as mean±standard error of the mean. *p<.05 compared with IL-1β–pretreated cells with n=5 in each group. ADAMTS4, a disintegrin and metalloproteinase with thrombospondin motifs; MMP9, matrix metalloproteinase-9; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; COX-2, cyclooxgenase-2; MMP13, matrix metalloproteinase-13; IL-6, interleukin-6; IL-8, interleukin-8; MMP3, matrix metalloproteinase-3.

as early as 60 minutes, with the main mechanism being necrosis [19]. More evidence for the toxic effect of bupivacaine comes from studies on chondrocytes, in which 0.25% bupivacaine resulted in 41% reduced viability after 15 minutes already, whereas a concentration of 0.125% was not cytotoxic [15]. The findings of our study and the studies mentioned above provide critical new information relating to current concerns on the clinical use of bupivacaine. From Lee's study, it becomes evident that both nucleus pulposus and annulus fibrosus cells showed increased cell death after bupivacaine treatment [19]. In our study, cells from the entire disc were used as degeneration was progressed so far that distinguishing between the two regions was not possible anymore. Based on the studies done on IVD cells so far, it is unclear whether the cells from degenerated discs are more susceptible than the cells from healthy discs. However, studies on chondrocytes indicate that an intact articular surface has a chondroprotective effect [16], thus providing evidence that diseased tissue shows increased drug susceptibility.

Interestingly, cytotoxicity was replaced by stimulation of cell proliferation at lower concentrations (0.1–1.0 mmol), especially after 18 hours. The minor effects after 2 hours are understandable, as cell growth requires adequate times. To the authors' knowledge, this is the first study demonstrating that bupivacaine can exhibit either a cytotoxic or a proliferative effect in IVD cells, depending on the concentration used. A proliferative effect of bupivacaine at concentrations lower than 0.1 mmol could also be observed in keratinocytes, whereas high concentrations showed severe toxicity in this cell type as well [21]. The proliferative effect could be a beneficial aspect for the treatment of degenerated disc disease. As the IVD is characterized by low cell numbers [22] with reduced functionality (which may lead to typical signs of degenerative disc disease), a proliferative side effect of an intradiscally applied drug could possibly help counteracting the process of degeneration.

The major issue of cell viability measurements in an in vitro cell culture model is the questionable direct

transferability to the in vivo situation. The reason is that it is unclear how quickly the drug is cleared from the tissue because of diffusion and bulk flow of fluids. Additionally, the volume of the disc will cause a certain dilution, and the extracellular matrix, which was missing in our cell culture study, may have a strong influence on the bioavailability. However, there is certain evidence from the latest research indicating that data from in vitro cell culture studies can be confirmed in tissue explant studies, in which cells are at least protected by the native matrix. So far unpublished data on organ culture testing of bupivacaine were presented at the 2010 International Society for the Study of the Lumbar Spine Meeting, showing that the cytotoxic effect previously observed on isolated cells could also be observed in excised mouse disc tissue [23]. The cell and tissue culture studies performed in the most recent past clearly provide enough relevant data indicating that the next logical step would be to follow up with an in vivo animal study, the most valuable system for drug testing.

For this study, we used a self-made sterile stock solution of bupivacaine rather than a commercially available solution, which has advantages and disadvantages. With our resources, we are not able to ascertain that in the process of preparing the sterile bupivacaine stock solution, no toxic byproducts have arisen. However, one of the most relevant anesthesiology textbooks clearly states that amide-based anesthetics such as bupivacaine are not very susceptible to heat and can thus be heat sterilized [24]. As a major advantage compared with commercially available bupivacaine solutions, no pH adjustment was necessary in this stock that was always used fresh, therefore minimizing any pH-related cytotoxic side effects that could arise in commercial preparations (pH 4.0–4.4).

Gene expression

We hypothesized that bupivacaine may also have an effect on inflammatory and catabolic mediators at nontoxic concentrations. So far, there is no information about the inflammatory or catabolic response of disc cells to bupivacaine treatment, and studies on other types of cells or tissues are rare and very contradictive [7,25–29]. In our study, we used IL-1β prestimulation to simulate the situation during IVD disease, as IL-1β is known to cause reliable catabolic and inflammatory effects. Although treatment with IL-1β is commonly used for this purpose, it can only simulate certain aspects of the inflammatory situation in the IVD in vivo. Treating prestimulated cells with a nontoxic concentration of bupivacaine resulted in a partial reduction of TNF-α and COX-2 expression, thus indicating its anti-inflammatory effect. Importantly, it also significantly reduced the expression of several relevant matrix-degrading enzymes in the disc, even at 2 hours. Although the observed effects may be clinically interesting, it is possible that repetitive injection would be necessary. However, repetitive disc puncture may entail negative side effects that need to be investigated before announcing a novel therapeutic value of bupivacaine. The positive effect of bupivacaine with regard to mRNA levels is further limited because of a slight increase in the expression of MMP3 and a strong increase in the expression of IL-1β (both after 18 hours). This negative effect was not present after 2 hours, but it is unclear how the expression of these genes would change with more time, whether changes would be translated onto the protein level, and for how long bupivacaine would influence cells in vivo at all. If the upregulation of IL-1β and MMP3 would be maintained long enough in vivo, induction of degradation and inflammation would be a matter of concern. Therefore, the expression pattern of IL-1β and MMP3 will have to be investigated in more detail.

Clinical relevance

If toxicity results obtained in this in vitro cell culture study can be corroborated in vivo (eg, using an animal model), caution will be necessary when applying bupivacaine as an adjunct during provocative discography with the currently used concentration.

Lower concentrations, which stimulated cell proliferation and reduced gene expression of certain matrix-degrading enzymes and proinflammatory cytokines, may be therapeutically beneficial, but more data are required to assure clinical purpose and safety.

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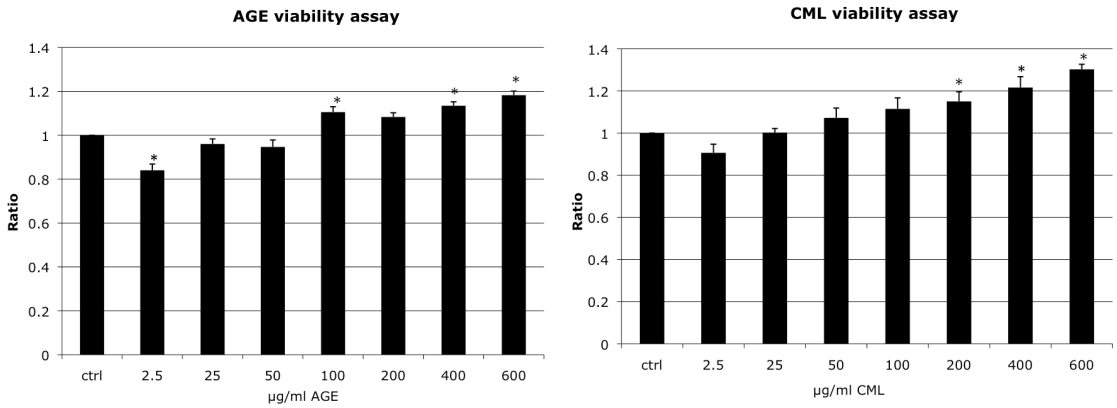
7. UNPUBLISHED DATA

7.1. AGE as a pro-inflammatory and catabolic mediator on IVD cells *in vitro*

AGE and its receptor RAGE are found to be increased during IVD degeneration [46, 47]. AGE is a non-enzymatically modificated tissue protein produced by the reaction of glucose with protein amino groups. These products are implicated in diseases such as diabetes. A study by Yoshida *et al.* [2009] could demonstrate a catabolic effect of AGE in combination with IL-1β. IL-1β decreased aggrecan mRNA level in bovine IVDs only in combination with AGE which was recovered by blocking RAGE activity [254].

We were interested in whether the accumulation of AGE can lead to a pro-inflammatory and catabolic stimulus in IVD cells *in vitro*. When investigating cell viability upon treatment with AGE or CML, no toxic effect was observed in the tested concentration range of 2.5 to 600 μg/ml after 18 hours (Figure 1A). On the contrary, it caused a minor proliferative effect. However, the stimulation of IVD cells with 200 μg/ml AGE and CML for 18 hours increased mRNA level of TNF-α, IL-8 and MMP13 (Figure 1B), but this was not statistical significant due to high patient-variation, i.e. only few donors were responsive to the stimulus. No effect was seen for all patients on investigated genes AD5, MMP1, MMP2, MMP13 and IL-6 (data not shown). For further investigations, different time points and concentrations have to be included.

A



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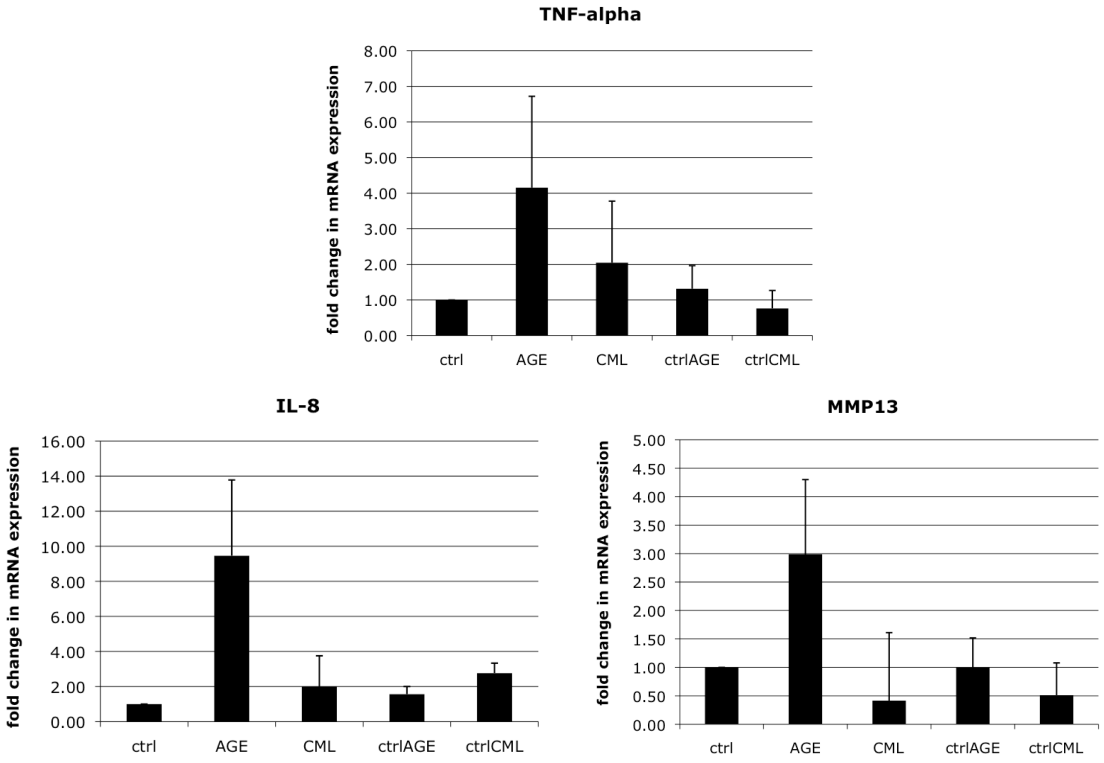


Figure 1A-B. Viability assay of AGE and CML on IVD cells (A) (n=3, Mean ± SEM). Gene expression levels of IVD cells treated with 200 µg/ml AGE and CML for 18 hours (B) (n=8, Mean ± SEM). Cells treated with BSA served as a control (ctrlAGE as well as ctrlCML). Statistical analysis was perfomed using student's t-test with a significant level of $p<0.05$.

7.1.1. Methods

AGE was prepared by dissolving of 100 mg of BSA (Sigma) and 750 mg D-Ribose (Sigma) in 50 ml PBS, pH 7.4 and incubated for 5 weeks at 37°C and 5% CO2 and dialysed to PBS. CML was generated by 100 mg BSA and 100 mg glycoaldehyde dimer (Fluka), but only incubated for 3 days and also dialysed to PBS. BSA alone served as a control. IVD cells were cultured in 2D monolayer in 150 ccm flasks. Before starting the experiment, cells were rendered serum free for 2 hours and then treated for 18 hours with 200 µg/ml AGE, CML or BSA alone as a control. For gene expression analysis, cells were harvested using trypsin treatment and mRNA was isolated using the mRNA Purification Kit according to the manufacturer's recommendation (Invitrogen). Thereafter, 1 µg of mRNA was reverse transcribed to cDNA (TaqMan, Applied Biosystems) and then used for real-time RT-PCR measurements using TaqMan Gene Expression assays (Applied Biosystems) for detection of cytokines (IL-6, IL-8, TNF-α), matrix degrading enzymes (MMP1, MMP3, MMP13) and aggreccanases AD4 and AD5. Gene expression was first normalized to the housekeeping gene TATA-Box binding Protein (TBP) before comparing expression of treated cells to untreated control (2-ΔΔCt method). Statistical analysis was performed using the student's t-test, with a significance level of $p < 0.05$.

7.2. Comparison of cytokine level of biopsies from disc herniation and trauma patients and from cells when cultured

As cytokines play a major role in pain development, we were interested in differences of cytokine levels from biopsies of herniated disc tissue to trauma patients. Therefore mRNA was isolated directly from biopsies. Gene expression level was measured by real-time RT-PCR and compared to the lowest expressed which was in both cases TNF−α followed by IL-6 and IL-1β (Figure 2). A huge increase we detected for IL-8 (45 time higher) in trauma patients compared to disc herniation patients, obviously giving IL-8 a tremendous role in acute tissue injury.

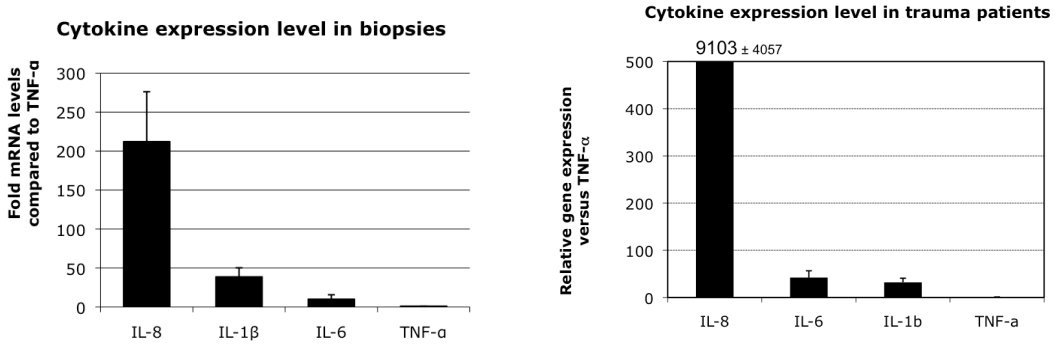


Figure 2. Comparison of mRNA cytokine levels from biopsies of herniated disc (n=5, left) and trauma patient (n=8, right). Gene expression was first normalized to the housekeeping gene TATA-Box binding Protein (TBP) before comparing expression to lowest expressed cytokine (TNF-α) which was set as 1 (Mean ± SEM).

Further we were interested in whether the cytokine level changes in IVD cells during culturing. Therefore, cells from biopsies of herniated disc were additionally expanded in a 2D monolayer culture system up to passage 1 and passage 3 where the experiments are generally conducted. First of all, measurement of mRNA level of cultured IVD cells revealed that IL-8 was no longer the highest expressed cytokine like in biopsy but shifted to IL-6, more dominant in passage 3 (Figure 3).

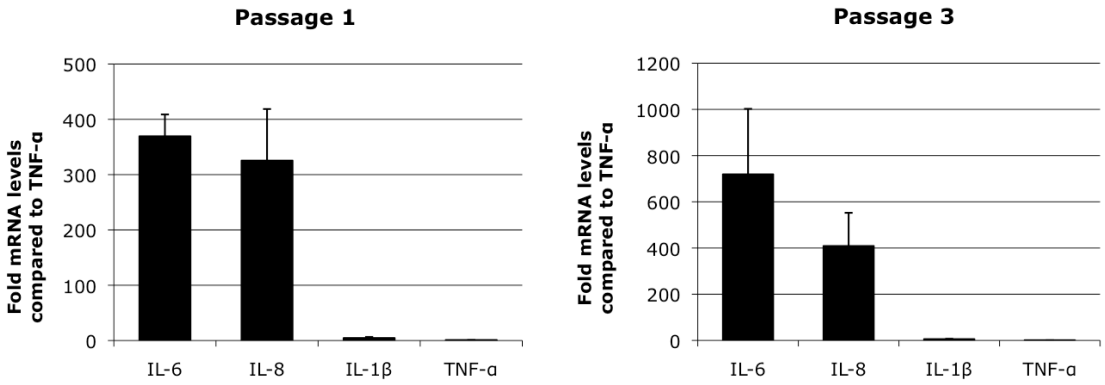


Figure 3. Gene expression profile of cytokines TNF-α, IL-1β, IL-6 and IL-8 in passage 1 (left) and passage 3 (right) of 2D monolayer cultured IVD cells from biopsies of herniated disc (n=5). Gene expression was first normalized to the housekeeping gene TATA-Box binding Protein (TBP) before comparing expression to lowest expressed cytokine (TNF-α) (Mean ± SEM).

When we compared gene expression level of cultured IVD cells to basal level of biopsy, we observation a huge drop of IL-8 mRNA expression (-1500 fold) which was not so dominant for IL-6 (-20 fold). IL-1β and TNF-α mRNA level decreases as well dramatically when IVD cells are cultured (Figure 4).

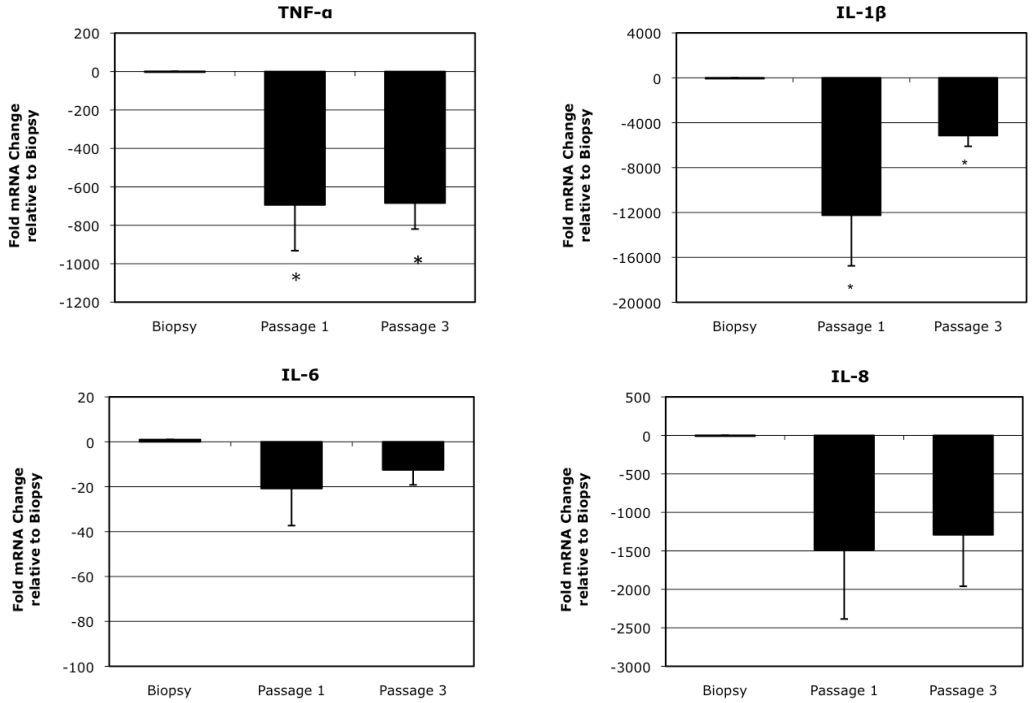


Figure 4. Change of mRNA level of cytokine TNF-α, IL-1β, IL-6 and IL-8 in 2D monolayer cultured IVD cells compared to cells from biopsy. Gene expression was first normalized to the housekeeping gene TATA-Box binding Protein (TBP) before comparing expression to biopsy which which was set as 1 (n=5, Mean ± SEM). Student's t-test was considered statistically significant at a level of $p<0.05$.

Due to the interesting observation that the mRNA cytokine level drops drastically when cells from IVD biopsies are in culture, we wanted to find optimal conditions for culturing IVD cells which is comparable to the biopsy environment concerning cytokine expression level. For testing different culture conditions, cells were isolated from biopsies and cultured either in 2D monolayer system or 3D (alginate beads) under normal condition (DMEM/F12, 10% FCS, 1% antibiotics, 37°C, 5% CO_2) or in a "microenvironment" (low glucose DMEM/F12, 10% FCS, 1% antibiotics, 37°C, 400 mOsm, pH 6.5) (Figure 5). mRNA was isolated either directly from biopsy or from cells expanded in the different culture conditions and cytokine gene expression was compared after two weeks of culturing.

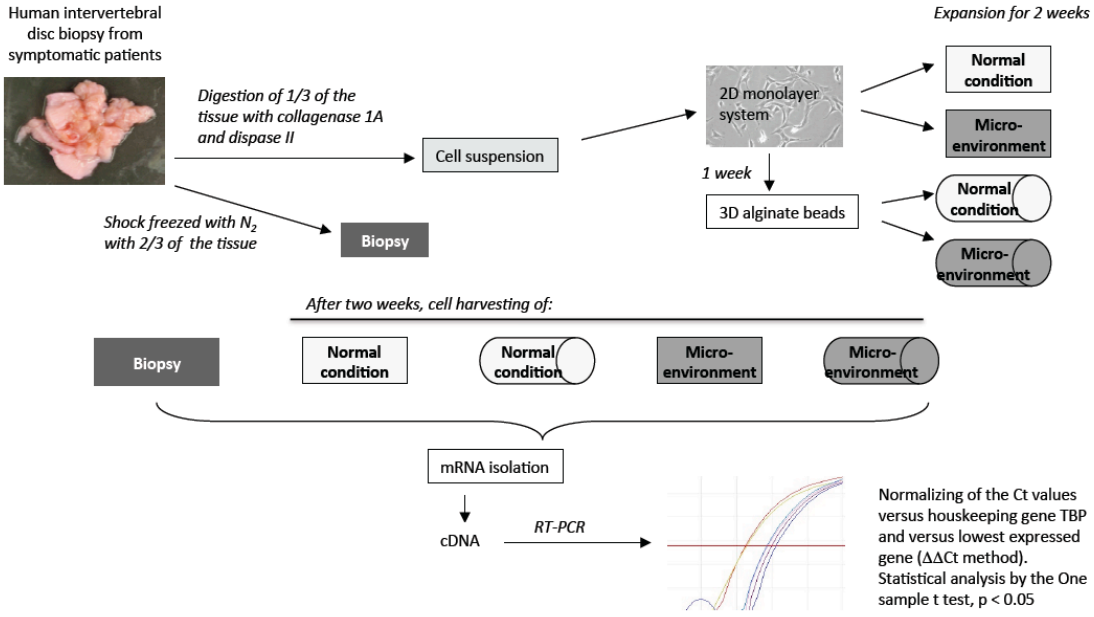


Figure 5. Schematic representation of conducted experiment for the evaluation of optimal culture condition of IVD cells, to regain cytokine mRNA level measured in biopsy.

Monitored mRNA level of these four different conditions revealed that already culturing of IVD cells in a "microenvironment" with low glucose and low pH contribute to the regain of the mRNA cytokine level detected in biopsy. This observation is also true when cells are cultured in a 3D system for TNF-α, IL-6 and IL-8 but not IL-1β. The most adequate culture method for IVD is the combination of both conditions with the reach of almost biopsy mRNA level for all measured cytokines (Figure 6).

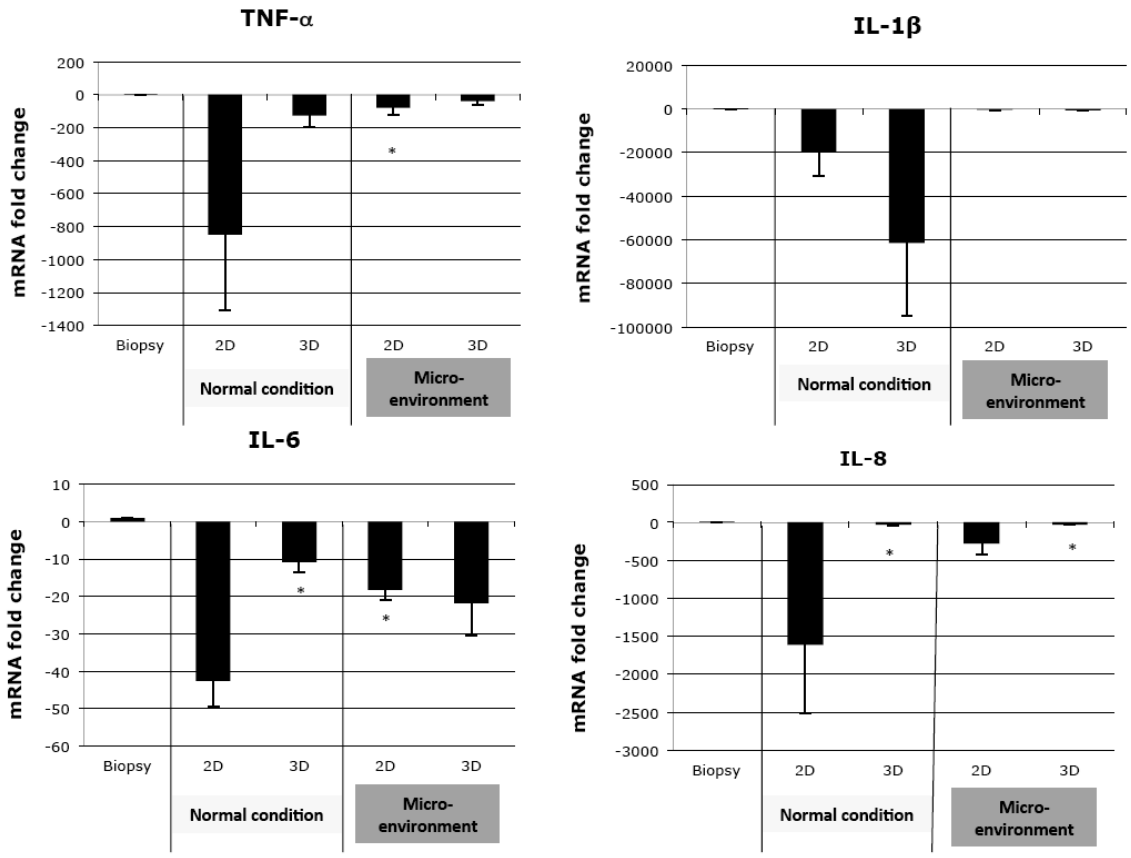


Figure 6. Comparison of mRNA level of cytokine TNF-α, IL-1β, IL-6 and IL-8 in 2D monolayer system, 3D system (alginate beads), under normal condition (DMEM/F12, 10% FCS, 1% antibiotics, 37°C, 5% CO2) or in a "microenvironment" (low glucose DMEM/F12, 10% FCS, 1% antibiotics, 37°C, 400 mOsm, pH 6.5) to cells from biopsy. Gene expression was first normalized to the housekeeping gene TATA-Box binding Protein (TBP) before comparing expression to biopsy which was as 1 (n=2, Mean ± SEM). Student's t-test was considered statistically significant at a level of $p<0.05$.

7.2.1. Methods

For the comparison of cytokine gene expression levels of biopsies of herniated disc to trauma patients, mRNA was isolated by TRI Reagent (Sigma) and 1-Bromo-3-Chloropropane (BCP, Sigma) followed by using the RNA Purification Kit (Invitrogen). In order to compare the cytokine levels of biopsies to cultured IVD cells (from the same biopsies), isolated disc cells were either cultured up to passage 1 or passage 3 before isolating mRNA with the RNA Purification Kit. For evaluating culture condition which is comparable to biopsy by comparing cytokine level, cells from biopsy of herniated disc were first expanded either to 70% confluency (for subsequent 2D culturing) or to 100% confluency (for subsequent 3D system) under normal condition (DMEM/F12, 10% FCS, 1% antibiotics, 37°C, 5% CO_2). Afterwards, the same amount of cells was cultured for 2 weeks in either a 2D monolayer or 3D (alginate beads) system under normal conditions or in a "disc-typical microenvironment" (low glucose DMEM/F12, 10% FCS, 1% antibiotics, 37°C, 400 mOsm, pH 6.5). mRNA isolation from cells expanded in the different culture conditions was conducted by mRNA Purification Kit. 1 µg mRNA was reverse transcribed to cDNA (TaqMan, Applied Biosystems) and then used for real-time RT-PCR measurements using TaqMan Gene Expression assays (Applied Biosystems) for detection of cytokines (IL-6, IL-8, TNF-α, IL-1β). Gene expression was first normalized to the housekeeping gene TATA-Box binding Protein (TBP) before comparing all mRNA level to either lowest expressed cytokine (TNF-α) or to biopsy cytokine level (2-ΔΔCt method). Statistical analysis was performed using the student's t-test, with a significance level of $p < 0.05$.

7.3. Receptor detection of TLR2, TLR4, CD44 and RHAMM in IVD cells *in vitro*

For monitoring gene expression levels of receptors on IVD cells investigated in fHA experiments *in vitro*, mRNA level was first measured of TLR2, TLR4, CD44 and RHAMM/(IHABP) of cultured IVD cells by real-time RT-PCR. Comparison of ct values revealed that RHAMM and TLR4 were most expressed (both 27%), followed by TLR2 (24%) and CD44 (22%) (Figure 7).

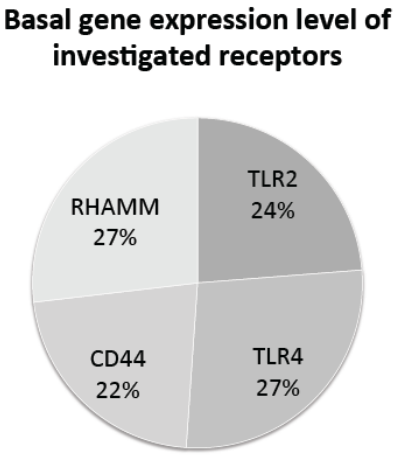


Figure 7. Comparison of basal gene expression profile of investigated receptors TLR2, TLR4, CD44 and RHAMM/(IHABP) in culture IVD cells.

Protein detection by wester blot of whole cell extract of cultured IVD cells revealed a successful staining of RHAMM/(IHABP) at already low applied amount of cells (ca. 2000 cells) followed by CD44 (ca. 30'000 cells). Only a faint staining for TLR4 (250'000 cells) was visible on the same level of the signal of recombinant TLR4 (positive control), but no clear TLR2 staining was detectable. Also TNF-α stimulated IVD cells (served as positive control) did not show any signal for TLR2 (Figure 8).

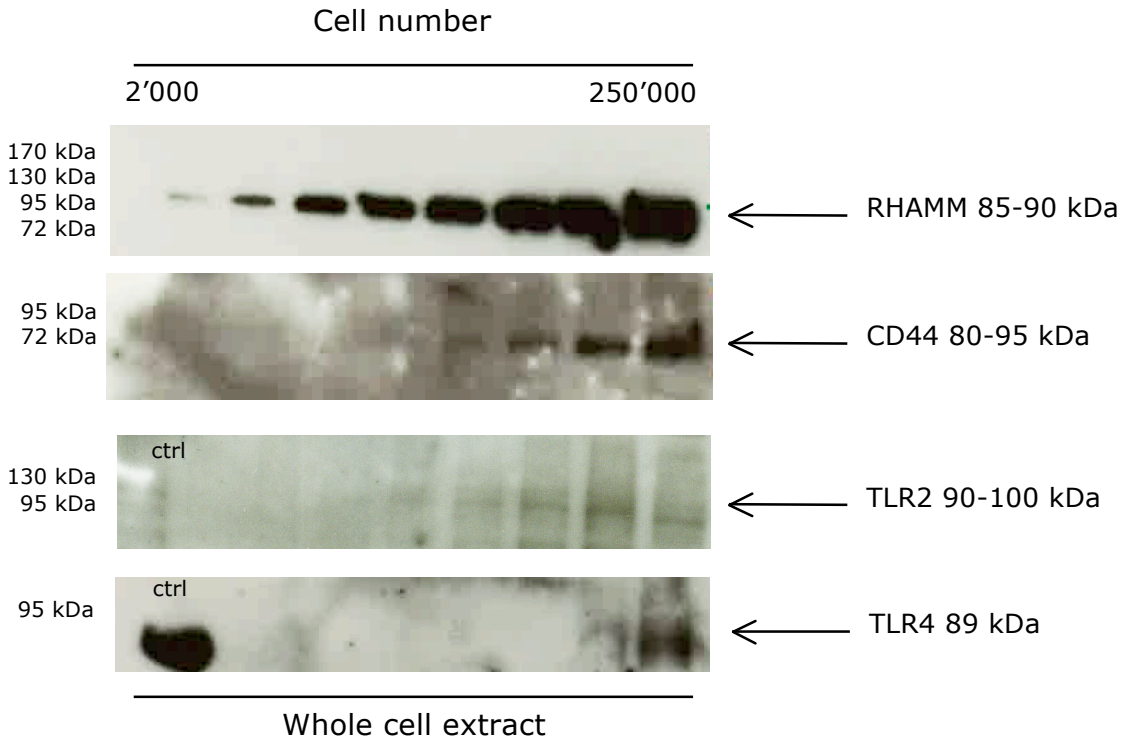


Figure 8. Receptor detection by Western blot of RHAMM/(IHABP), CD44, TLR2 and TLR4 in whole cell extract of cultured IVD cells.

Surface protein expression detection was achieved by FACS analysis. As a positive control, fresh isolated peripheral blood mononuclear cells (PBMC) were used for TLR2, TLR4 and CD44, MDA-MB-231 cell line was used for RHAMM. All used antibodies revealed a positive signal (Figure 9).

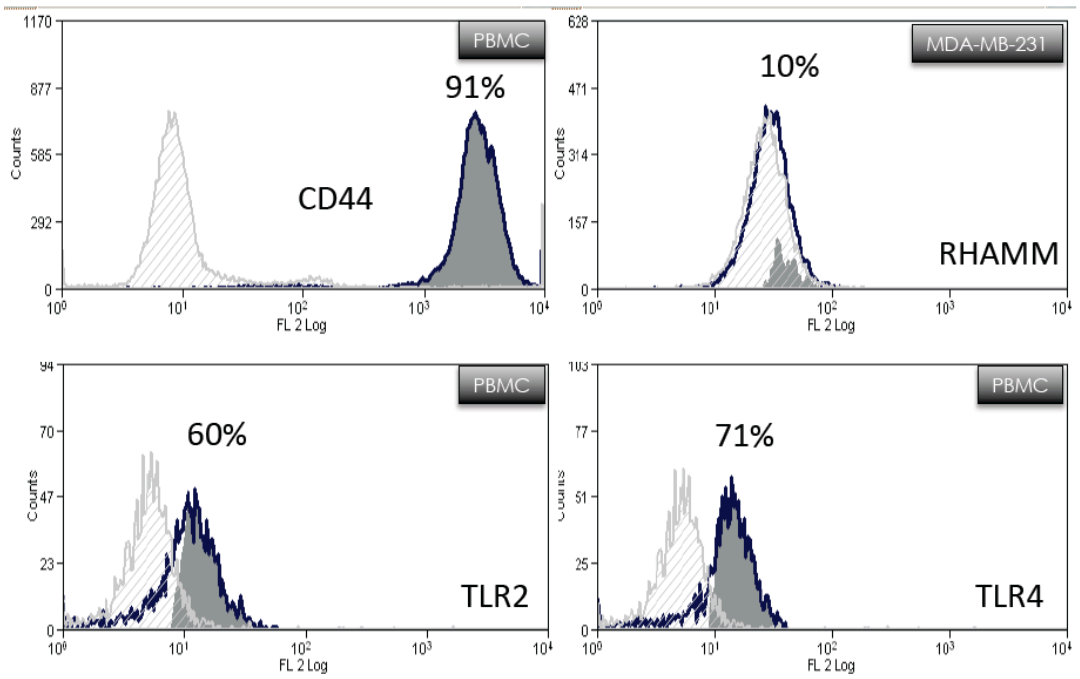


Figure 9. Monitoring of surface-receptor staining (black curve) by FACS analyis of TLR2, TLR4 and CD44 by using PBMC and RHAMM by using MDA-MB-231 cell line as positive control cells. Respective IgG antibody (grey curve) served as control for unspecific binding. The grey area represents the amount of positive stained cells, indicated as percentage.

RHAMM antibody was further evaluated by staining intracellular RHAMM/(IHABP) by first permeabilizing MDA-MB-231 cells. FACS analysis revealed 30% staining by additional intracellular RHAMM=IHABP detection (Figure 10).

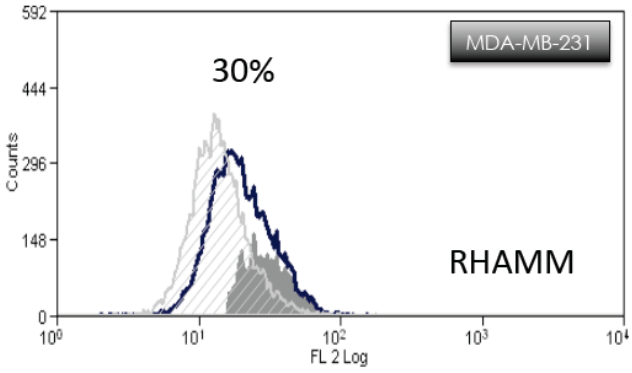


Figure 10. Permeabilized MDA-MB-231 cells for monitoring additional intracellular RHAMM/(IHABP) (black curve) by FACS analysis to control antibody staining. Respective IgG antibody (grey curve) served as control for unspecific binding. The grey area represents the amount of positive stained cells, indicated as percentage.

Staining of investigated cell surface receptor on cultured IVD cells was only positive for CD44 (95%), but no signal was detected for TLR2, TLR4 as well as for RHAMM (Figure 11).

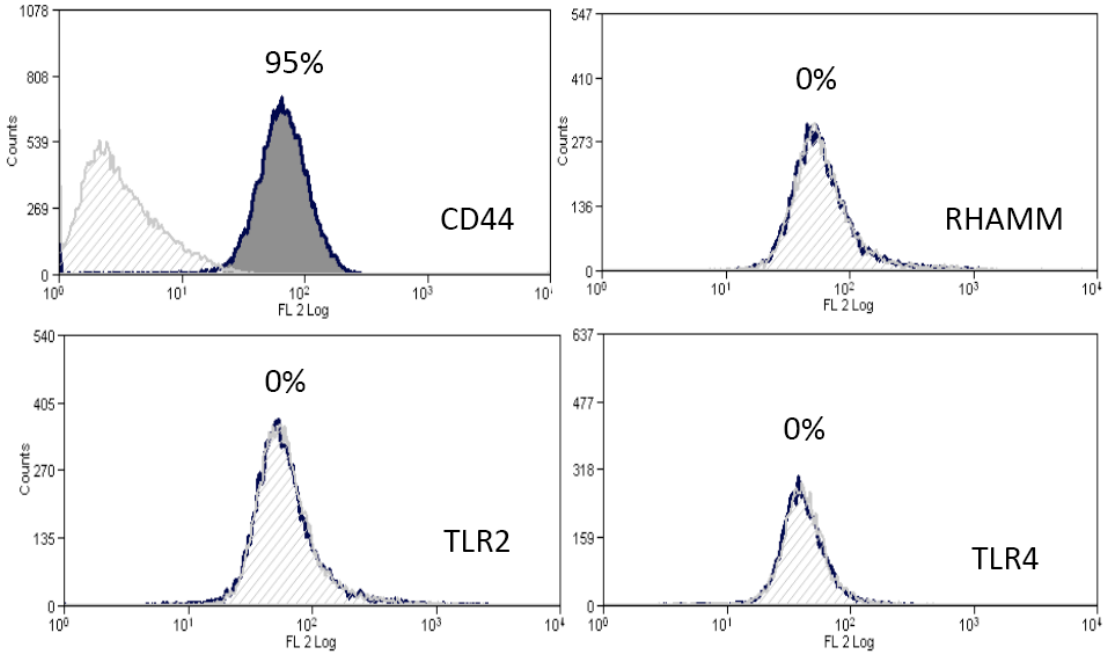


Figure 11. Cell surface-receptor stainig (black curve) of CD44, RHAMM, TLR2 and TLR4 of cultured IVD cells by FACS analysis. Respective IgG antibody (grey curve) served as control for unspecific binding. The grey area represents the amount of positive stained cells, indicated as percentage.

Receptors were additionally stained by immunofluorescence. Unfortunately, none of the used antibodies (all from abcam) was able to stain receptors in IVD cells as well as not in cells used as positive control (PBMC and MSC for TLR2, TLR4 and MDA-MB-231 for RHAMM), except CD44 (Biolegend, data not shown).

7.3.1. Methods

For comparison of receptor gene expression levels, mRNA of cultured IVD cells was isolated by mRNA Purification Kit, 1 µg/ml reverse transcribed and cDNA measured by real-time RT-PCR using TaqMan Gene Expression assays (Applied Biosystem).

For detection of receptors on protein level by Western blot, IVD cells were harvested by trypsin, collected and washed with PBS buffer. Cells were lysed with lysis buffer containing 50 mM HEPES (pH 7.5), 450 mM NaCl, 15% glycerol, 2 mM EDTA, 1 mM PMSF with freshly added 0.1% protease inhibitors pepstatin-A, leupeptin and bestatin, incubated on shaker for 10 min at 4°C and centrifuged for 30 min at 14'000 rpm. Supernatant was collected and protein concentration measured by Bradford assay and used for Western blot. Used antibodies were RHAMM (GeneTex), TLR2, TRL4 (GeneTex, Invivogen and R&D) and CD44 (Biolegend). Applied respective secondary antibody was HRP labeled and after washing, chemiluminescence was detected using SuperSignal West Dura on Hyperfilm.

For surface receptor staining, FACS analysis was perfomed. Therefore cultured IVD cells were harvested by scraping, pooled and washed with PBS. Cells were resuspended in FACS buffer (PBS/BSA 1%) at 1x10^6 cells/ml and distributed in a 96 well plate. After centrifugation, cells were resuspended in PBS/BSA 2% with additional respective serum (5% v/v) to block unspesific binding and incubated for 20 min at 4°C. After washing three time with cold FACS buffer, PE conjugated antibody was applied for 30 min at 4°C in the dark, washed again three times, resuspended in 400 µl 4% paraformaldehyd (PFA) in PBS and transferred to FACS tubes. For additional intracellular RHAMM/IHABP staining, cells were prefixed for 10 min with 4% PFA before permeabilizing with 0.1% Triton-X100 (Fluka) in FACS buffer for 10 min. Used antibodies RHAMM (antibodies-online.com), TLR2 and TLR4 (Biolegend) were all PE conjugated, except CD44 (Biolegend), which was stained by applying ALEXA Fluor488 conjugated secondary antibody (Molecular probe). As negative control, the respective PE conjugated IgG (GeneTex and Biolegend) antibodies were used. Fluorescence was monitored on CyAn^{TM} with excitation-emission wavelength of 480-578 nm for PE, using Dako Summit software, v 4.3.

7.4. Further effects of fHA (6-12 ds) on cultured IVD cells

The detected increased IL-6 and MMP1 mRNA level upon fHA treatment of cultured IVD cells we could also monitor on protein level by Western blot using precipitated protein from the supernatant. We could already detect an increased IL-6 protein level after treatment of cells with 1 µg/ml fHA. MMP1 showed an increased signal after treatment with 50 µg/ml fHA (Figure 12).

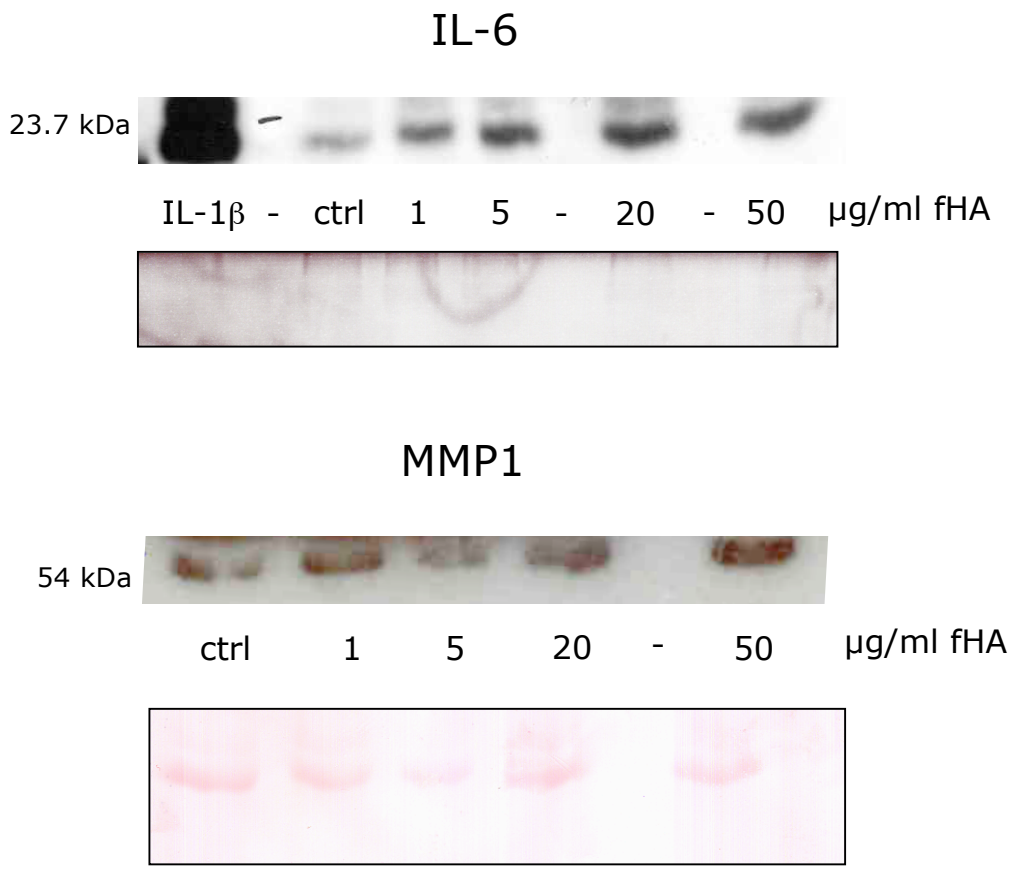


Figure 12. Staining of precipitated IL-6 and MMP1 of supernatant of IVD cells by Western blot (n=3, ctrl = untreated cells). Coomassie staining of SDS-gel served as loading control for IL-6, ponceau S staining of membrane served as loading control for MMP1.

Furthermore, fragments of CD44 were monitored in conditioned media of IVD cells. Whole cell extract (WCE) of HeLa cells was used as a control, where we could see a clear band on the expected size of 81.5 kDa. In the supernatant of IVD treated cells instead we detected a smaller size which might be cleaved CD44 (55 kDa), already present at untreated cells with a slightly increased signal at cells treated with 50 μg/ml fHA (Figure 13). It has been reported that ECM fragments are able to provoke cleavage of CD44. This has been demonstrated for fHA 3-7 ds and 17 ds in tumor cells, where the membrane bound cleavage product of CD44 (25 kDa) was detected [203]. It might therefore be that fHA provokes the cleavage of CD44 in IVD cells.

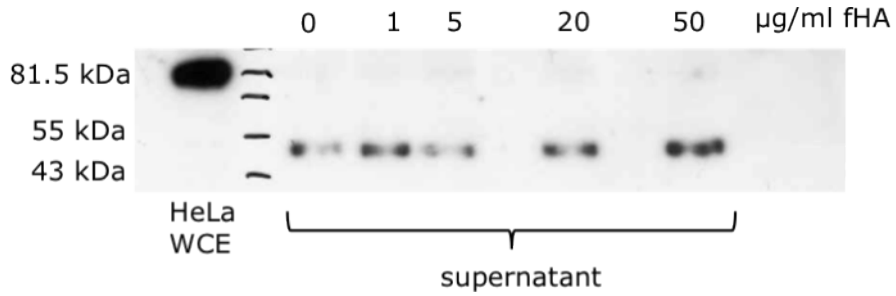


Figure 13. Staining of CD44 by Western blot in whole cell extract (WCE) of HeLa cells (as control) and in supernatant of fHA treated IVD cells. 81.5 kDa represents the full size of CD44, lower band might indicate cleaved CD44 product (near 55 kDa) (n=1).

Helenalin is promoted as a specific NF-κB inhibitor and claimed to specifically and irreversibly alkylating free sulfhydryls of the cystein residues on the NF-κB subunit p65 and thereby preventing the interaction with its target DNA sequence [255]. But it is also described to specifically inhibit activation of NF-κB by preventing the degradation of IκB-α and IκB-β [256]. It is used in different studies to investigate NF-κB dependent reactions where the monitored effects are claimed to be NF-κB dependent just due to the observed altered signals in helenalin treated cells [211, 213]. In our study, we revealed that helenalin indeed had inhibitory effects by decreasing IL-6 secretion in IL-1β as well as in fHA treated IVD cells (Figure 14), but as fHA had no effect on p65 shuttling into nucleus and obviously not activates NF-κB pathway (see results in fHA paper in

preparation), it seems that helenalin is targeting also other pathways involved in inflammatory signalling and is not restricted to the NF-κB pathway.

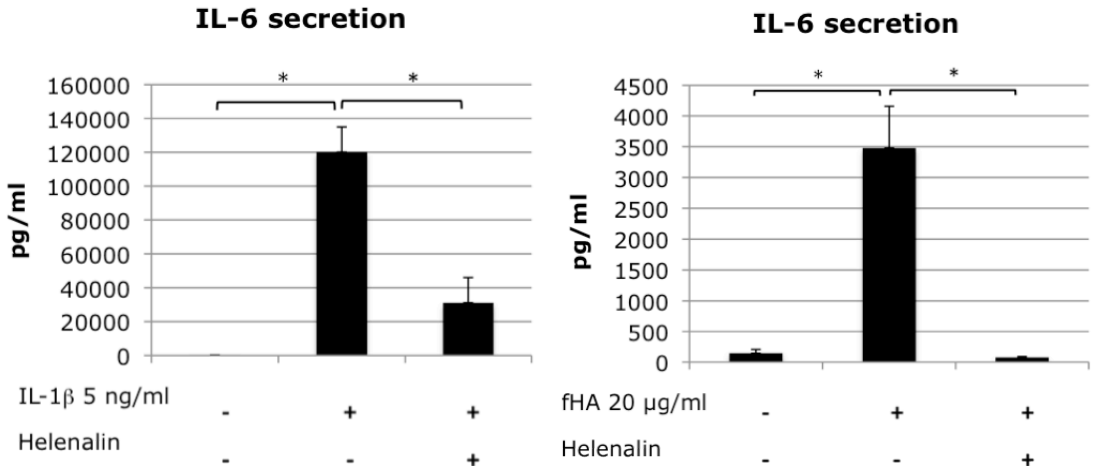


Figure 14. IL-1β provoked IL-6 protein production in IVD cells was downregulated by 1 µg/ml helenalin (left) as well as in fHA treated cells (right) (n=5, Mean ± SEM). Student's t-test was considered statistically significant at a level of $p<0.05$.

Western blot analysis revealed that helenalin did not prevent p65 nuclear shuttling in IL-1β treated IVD cells (Figure 15), indicating at least that helenalin does not intervene in this step of NF-κB activation by e.g inhibiting degradation of IκB as described by Hehner *et al.* [1998] [256], but may then rather inhibit binding of p65 on DNA as it is discussed by Lyss *et al.* [1997, 1998] [255, 257].

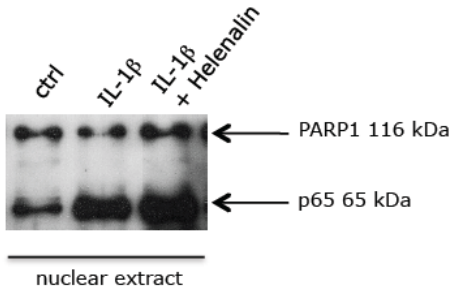


Figure 15. Staining of nuclear p65 by Western blot of IVD cells treated with 5 ng/ml IL-1β or thogether with 1 µg/ml helenalin (promoted as a specific NF-κB inhibitor) (n=2).

The 3D alginate bead system has shown to be an ideal environment for the study of IVD cells, at least concerning cytokine expression. Therefor we started conducting fHA experiments in IVD cells cultured in alginate beads. Viability assay indicated comparable toxic effects of 250 µg/ml fHA on IVD cells in the 3D culture system (Figure 16 right) to the 2D monolayer system (Figure 16 left). These are already promissing results to conduct future experiments in this 3D culture system as it represent the *in vivo* environment more closely than 2D culture.

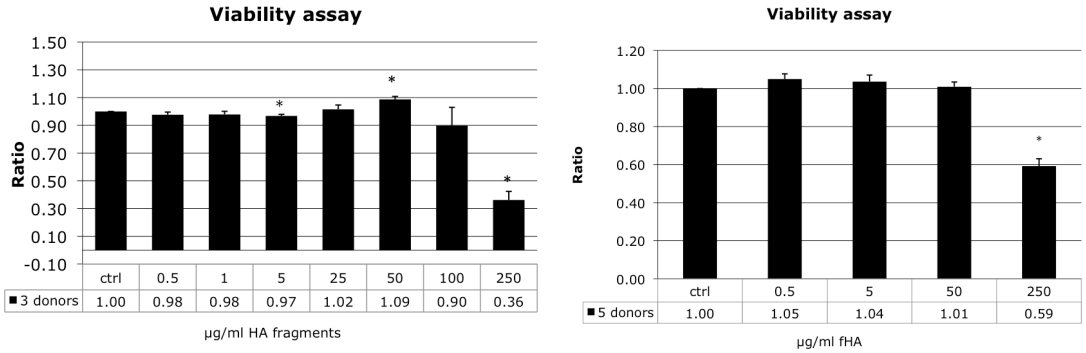


Figure 16. Viability assay for fHA (0-250 µg/ml) on IVD cells conducted in a 2D monolayer system (left, n=3) or in a 3D system (right, alginate beads, n=5, Mean ± SEM). Student's t-test was considered statistically significant at a level of $p<0.05$.

7.4.1. Methods

For Western blot analysis, conditioned media (10 ml) of cells in 150 ccm flasks was collected after the experiment and proteins were precipitated by adding 1/100 sodium deoxycholate for 30 min at 4°C and 1/100 of 100% trichloracetic acid o/n at 4°C. Pellet was resolved after centrifugation in lysis buffer (50 mM HEPES, pH 7.5, 420 mM NaCl, 0.5% NP-40, 15% glycerol, 2 mM EDTA, 1 mM PMSF with freshly added 0.1% protease inhibitors pepstatin-A, leupeptin and bestatin) by ultrasonication for a few seconds. Protein concentration was measured by using Bradford reagents and proteins were separated on a 12% polyacrylamid gel by SDS-PAGE and transferred to a PVDF membrane. After blocking with BSA, the membrane was incubated first with primary antibody for 2 hours against IL-6 (Sigma), MMP1 (GeneTex) and CD44 (GeneTex), washed three times with TBS-Tween 20 and incubated for 1 hour with the respective

secondary HRP labeled antibody. After washing, chemiluminescence was detected using SuperSignal West Dura on Hyperfilm. For loading control, SDS-gels were stained with coomassie brilliant blue (Fluka) and membrane with ponceau S (Fluka) (n=3).

In inhibition experiments with Helenalin (ENZO Life Science), cells were seeded in 12 well plates (130'000 cells/well) one day before treatment. Cells were pretreated with or without 1 μg/ml helenalin in serumfree media for 1 hour before adding 5 ng/ml IL-1β or 20 μg/ml fHA. After 18 hours, supernatant was used for ELISA measurements (n=5).

For pathway analysis by Western blot for staining nuclear p65, cells were treated the same like described above, but harvested after 1 hours by usage of a cell scraper, 2 wells per condition were pooled and washed with buffer A containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 5 mM DTT with freshly added 0.1% protease inhibitors pepstatin-A, leupeptin and bestatin and then lysed with 0.1% NP-40 for 5 min. After centrifugation at 10'000 rpm for 5 min at 4°C, supernatants were discarded and nuclear pellets were washed with 0.1% NP-40 and lysed for 20 min with 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM PMSF and 5 mM DTT. After centrifugation, supernatants were measured for nuclear protein concentration by Bradford assay and stored at -80°C. Western blotting was performed as already described with a first antibody against p65 (Santa Cruz) or PARP1 (Santa Cruz) used as a loading control (n=2).

2D viability assay was performed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For this, cells were seeded in 24-well plates (50'000 cells/well) or in alginate beads. The respective fHA concentrations were analyzed in triplicates for each donor. After 18 hours, cells were analyzed for cell proliferation and viability. A fresh sterile solution of MTT (Sigma, Switzerland) with a concentration of 0.5 mg/ml in DMEM/F12 medium was prepared, 500 μl added to each well and incubated for 3 hours at 37°C. MTT was discarded, cells were lysed with dimethyl sulfoxide for 10 minutes at 37°C, and absorbance was measured at 565 nm. Absorbance of treated cells was calculated relative to absorbance of untreated control cells, which is set to 1 (n=3).

3D viability assay was performed by counting dead cells stained with trypan blue by using a hemocytometer (n=5).

7.5. Effects of a mixture of larger size of fHA < 25 ds on cultured IVD cells

We also tested pro-inflammatory and catabolic effects on IVD cells in culture of a mixture of larger size of fHA < 25 ds. Interestingly, results of 18 hours treatment with different concentration indicated that IVD cells responded to fHA < 25 ds at only low dose (1 μg/ml) monitored by the increased mRNA level of IL-6 (28 fold, Figure 17 left), MMP1 (8 fold) and MMP3 (9 fold) (data not shown). We also observed minor increase of MMP13 (3.7 fold) and IL-8 (2.5 fold) but no increase of COX-2, TLR2, TLR3, TLR4, IL-1β, Hyal1 and Hyal2 (data not shown) (n=2). On protein level, we also saw this dose dependency, with higher secretion of IL-6 measured at lower dose.

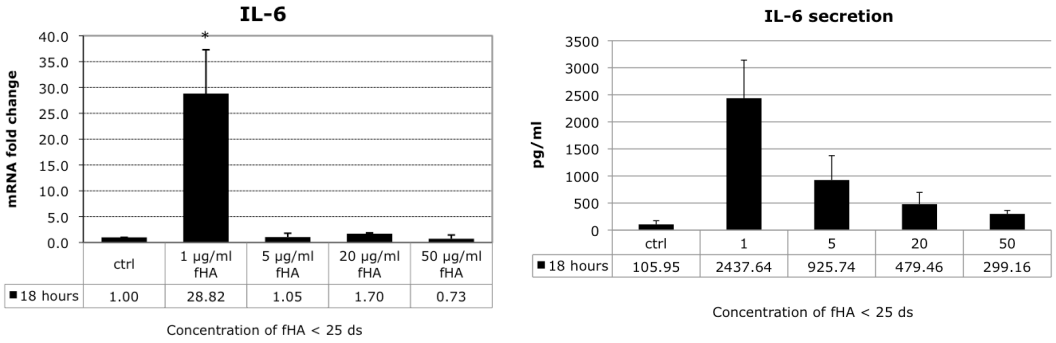
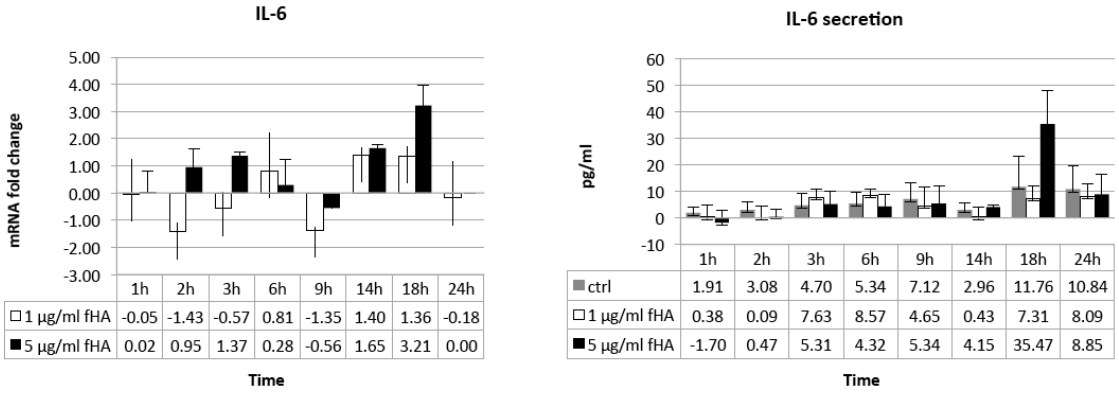


Figure 17. Effects of different concentration of a mixture (size) of fHA < 25 ds on IVD cells after 18 hours on gene expression (left, measured by real-time RT-PCR) and protein expression (right, measured by ELISA with precipitated protein of supernatant) (n=2, Mean ± SEM). Student's t-test was considered statistically significant at a level of $p<0.05$.

We also performed a time course and treated IVD cells with 1 and 5 μg/ml fHA < 25 ds for 1, 2, 3, 6, 9, 14, 18, and 24 hours (n=2). Again, only after 18 hours we saw an increase of mRNA level of IL-6 (Figure 18 left), also detected on protein level (Figure 18 right), but only with 5 μg/ml fHA < 25 ds. The increase was not statistical significant. This would indicate a greater inflammatory and catabolic potency of smaller fragments on IVD cells which we saw with fHA = 6-12 ds treatment. (see results in fHA paper in preparation).



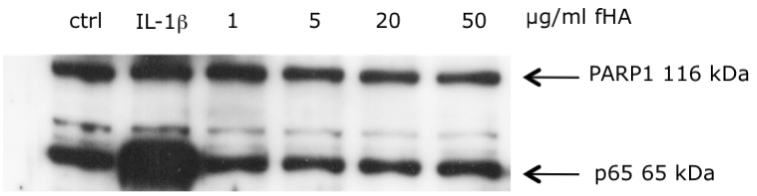
	1h	2h	3h	6h	9h	14h	18h	24h
1 μg/ml fHA	-0.05	-1.43	-0.57	0.81	-1.35	1.40	1.36	-0.18
5 μg/ml fHA	0.02	0.95	1.37	0.28	-0.56	1.65	3.21	0.00

	1h	2h	3h	6h	9h	14h	18h	24h
ctrl	1.91	3.08	4.70	5.34	7.12	2.96	11.76	10.84
1 μg/ml fHA	0.38	0.09	7.63	8.57	4.65	0.43	7.31	8.09
5 μg/ml fHA	-1.70	0.47	5.31	4.32	5.34	4.15	35.47	8.85

Figure 18. Effects of 1 and 5 μg/ml of a mixture (size) of fHA < 25 ds on IVD cells after different time points on gene expression (left, measured by real-time RT-PCR) and protein expression (right, measured by ELISA of directly applied supernatant) (n=2, Mean ± SEM). Student's t-test was considered statistically significant at a level of $p<0.05$.

Pathway analysis also indicated a NF-κB independent action of fHA < 25 ds, evaluated by Western blot (Figure 19A) and NF-κB binding assay (Figure 19B), which we also observed in cells treated with small fHA (6-12 ds, see results in fHA paper in preparation). The MAP kinase p38 was not activated by fHA < 25 ds (Figure 19C), which we could see partially with small fHA.

A



B

C

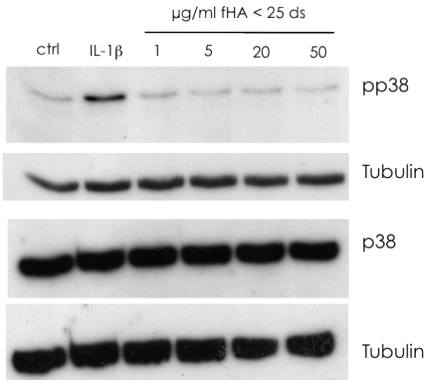


Figure 19 A-C. Pathway analysis of NF-κB and the MAP kinase p38 investigated in IVD cells treated with fHA < 25 ds. Nuclear p65 was stained by Western blot and signal was compared of untreated, IL-1β treated or fHA (1-50 µg/ml) treated cells, where PARP1 served as loading control (A, n=3). NF-κB binding activity was measured with a NF-κB transcription factor assay and absorption was recorded at λ=655 nm (B, n=2). MAP kinase p38 was monitored by staining of phosphorylated and unphosphorylated p38 with tubulin as additional loading control (C, n=2).

7.5.1. Methods

For the dose and time dependency experiments with mixed size fHA < 25 ds, cells were treated as already described. Cells were harvested at respective time points by TRI Reagent and proteins of supernatant were either first precipitated (dose dependent experiment) or directly used for ELISA (time course experiment).

Protein isolation of nuclear extract for NF-κB pathway analysis by immunoblotting or NF-kB (p65) Transcription Factor assay is described in chapter 7.4.1. Western blotting was performed as already described with a first antibody against p65 (Santa Cruz, 1:200) or PARP1 (Santa Cruz, 1:1000) used as a loading control (n=3).

For NF-κB (p65) Transcription Factor assay (n=2), which was used as a further analysis of NF-κB DNA binding, nuclear extracts were analysed using the NF-κB (p65) Transcription Factor Assay (Cayman, Estonia) as recommended by the manufacturer. Double stranded DNA (dsDNA) containing NF-κB binding site (coated on a 96 well plate) was incubated with the samples and specific binding was detected with an antibody against p65 as well as a secondary HRP conjugated antibody. Data analysis and quantification was performed by colorimetric measurement (655 nm).

Protein isolation of whole cell extracts for Western blot for p38 was performed as described in 7.3.1. and stained for phosphorylated or unphosphorylate p38 (1:500) (all Cell signaling) and tubulin (1:1000) (Cell signaling) (n=2).

DISCUSSION AND PERSPECTIVES

8. Summary of Results

8.1. fHA as a trigger of discogenic back pain?

Evidence from the literature suggests that symptomatic degenerated discs are characterized by elevated cytokine levels. These cytokines are thought to play a major role in the pain mediation by diffusing to the outer part of the disrupted disc tissue resulting in an irritation of nociceptors. In accordance to this hypothesis, pro-inflammatory cytokines within the NP tissue have been shown to evoke radicular pain via irritation of the DRG [67, 69, 70]. It is therefore of relevance to reveal the responsible factors which might provoke a pro-inflammatory cascade in a degenerated disc. Based on this knowledge, novel therapeutic approaches can be developed.

In the first part of this thesis, we were interested in identifying degeneration products with inflammatory and catabolic effects as these could possible contribute to the development to discogenic back pain and worsen the progress of IVD degeneration. During disc degeneration, there is an overall matrix breakdown, leading to an accumulation of certain ECM fragments with increased degeneration grade, which has been demonstrated so far for fibronectin [29, 31, 258] and aggrecan [27, 32]. HA is an important compound within the ECM of the IVD, and as a high molecular weight hyaluronic acid (HMWHA), together with other proteoglycans and glycosaminoglycans (GAGs), responsible for tissue hydration. HMWHA can be degraded to fragments either enzymatically by Hyals or by radicals occurring during oxidative stress. Furthermore, AGE and CML, which are shown to be increased in degenerated discs [46], may contribute to the fragmentation of HMWHA *in vivo* due to their radical nature as indicated by cell-free *in vitro* experiments [259].

In this work, we were able to show that fHA with a size of 2.4-4.6 kDa (6-12 ds, 12-24-mer) increased mRNA level of cytokines and MMPs in IVD cells *in vitro*, namely *IL-1β, IL-6, IL-8, MMP1*, *MMP3, MMP13* and *COX-2*. Results were furthermore verified on the protein level for IL-6 and MMP1. As COX-2 is an important enzyme for the generation

of prostaglandines, its increase might play a major role in pain sensation in symptomatic patients. Furthermore, the increased MMP levels observed upon fHA treatment could worsen the degenerative state, as MMPs are responsible for the degradation of structure proteins in the ECM.

IL-6 was traditionally considered as an activator of acute phase responses and a lymphocyte stimulatory factor [260]. Several studies conducted in animal models have now demonstrated a pain-mediating role for IL-6. As its levels are known to be elevated in symptomatic IVDs [60], we investigated the underlying pathway leading to the increased IL-6 secretion in IVD cells upon fHA treatment. Potentially involved receptors in this event are the main receptors of HA, namely RHAMM and CD44, as well as important receptors in immune reactions such as TLRs which are already shown to be engaged by fHA in several other cell types. In recent years, it has been recognized that TRLs are not restricted to immune cells, as they were also discovered to be expressed on synovial fibroblast [85-87], chondrocytes [83, 89] and hepatic cells [90]. Various studies investigating the reaction upon fHA exposure have demonstrated that depending on cell type and readout, the engaged TLR type varies. In dendritic cells for instance, fHA led to an increased TNF-α synthesis through TLR4 while being TLR2 independent [215]. In contrast, MIP-1α production was promoted through TLR2 in murine alveolar macrophages, but not through TLR4 [206]. Interestingly, TLR4 was responsible for the increased IL-8 synthesis but not for the increased MMP2 level due to fHA treatment in melanoma cells [207]. This fact is also pointing out the diversity of fHA engaged pathways, depending on the readout. In primary human chondrocytes which resemble the cells in center of the IVD, very small fHA induced an increase in IL-6, which was due to TLR4 and CD44 together with an involvement of the NF-κB pathway. However, in our study on human IVD cells, we revealed a significant dependency of TLR2 by knock-down experiments using siRNA. fHA induced IL-6 production was significantly decreased upon TLR2 gene silencing which could not be observed in TLR4, CD44 or RHAMM knock-down cells. TLR2 engagement was further proven through antibody-mediated inhibition studies, where functional loss of TLR2 activity led to a decrease in IL-6 production in cultured IVD cells. The involvement of NF-κB in mediating the effects of fHA has been well documented in multiple cell types [202, 206, 208, 211, 212].

Several studies have also investigated the MAP kinases in fHA treated cells and have identified p38, ERK as well as JNK to be activated by fHA. MAP kinases are involved in regulating cell cycle, differentiation and are important in immune and inflammatory responses due to extracellular stimuli (mitogens). In the present study, we were able to exclude NF-κB dependent signal transduction as being responsible for mediating the effects of fHA in IVD cells due the lack of any evidence of its activation following fHA stimulation. Instead, MAP kinases p38, ERK and JNK were all activated by fHA, as determined by Western blot analysis. Furthermore, inhibition of either ERK or JNK resulted in a significant decrease of fHA induced IL-6 secretion in IVD cells.

This is the first study demonstrating that fHA is a potential trigger of the pro-inflammatory cascade in primary human IVD cells *in vitro*. Similar to fibronectin and aggrecan, there is evidence that fragmentation of the HMWHA may also occur during aging in IVDs *in vivo*. During aging/degeneration of the IVD, an accumulation of AGE has been reported, which has been shown to play a role in depolymerization of HA in other tissues due to free radical related metabolism, e.g. in the vitreous body of the eye [261-264]. These non-enzymatic glycated proteins (AGE) have been proved to efficiently degrade HA *in vitro* [218, 259]. Furthermore, a free radical depolymerization of HA has been demonstrated in a murine keratinocyte cell line. The inhibition of ROS formation led to a decrease in fHA production together with a melioration of the inflamed condition [219]. The increased AGE and NO formation measured in degenerating IVDs might therefore trigger HA fragmentation under certain circumstances and would be interesting to investigate in the future.

The degree of accessible fHA engaged receptors might be of importance for the observed increase in IL-6 production and may therefore be of particular significance in the nociception in discogenic back pain. For instance, Campo *et al.* [2010] measured an elevated gene expression of the engaged receptor *TLR4* and *CD44* in human chondrocytes upon fHA treatment, which they also observed at protein level [208]. To evaluate changes in the expression pattern of receptors investigated in our fHA experiments, we conducted the detection of receptors in IVD cells by different methods. Gene expression analysis revealed that basal mRNA level were highest for RHAMM and TLR4, followed by TLR2 and CD44. Western blot analysis confirmed protein expression

of RHAMM and CD44, although TLR2 and TLR4 could not be identified. However, we were most interested in the basal amount of expressed surface-receptors on IVD cells, and thus performed FACS. The only receptor detectable was CD44, which was also confirmed by immunocytostaining. Although we were able to confirm their presence on PBMCs and MDA-MB-231 by FACS analysis, we were unable to detect RHAMM, TLR2 or TLR4 on IVD cells. PBMCs are generally known to express TLRs, whereas breast cancer cells like MDA-MB-231 are characterized by elevated RHAMM basal level. It might therefore be that the basal expression level of surface RHAMM receptors as well as of TLRs on IVDs were below the detection limit. Alternatively, the method of analysis may need to be optimized as it is reported (e.g. for TLR4) that cell surface receptors can be internalized immediately upon engagement, which might have occured during FACS preparation. RHAMM is discussed to be a non-integral cell surface hyaluronan receptor [177], and thus it might be that the preparation for FACS analysis destroys its interaction with the cell surface on IVD cells. However, there are no study available so far that has successfully shown cell surface-receptor staining of RHAMM and TLRs on IVD cells.

We could further demonstrate that the inflammatory fHA potency might be size dependent, as a mixture of larger sized fragments (< 25 ds) did not induce such a noticeable increase in IL-6 production like the small fHA (6-12 ds), and in some patients had no effect at all. Thus, this may have significant implications with regard to the involvement of fHA in IVD degeneration, assuming that there exists a disproportional amount of smaller fHA as compared to larger fHA in symptomatic degenerated discs. There are also several studies demonstrating size dependent key functions, reviewed by Stern *et al.* [2006] [265].

8.2. Anti-inflammatory and anti-catabolic effects of Resveratrol, Curcuma and Triptolide

Current treatment options for discogenic back pain involve either pharmacological agents, physiotherapy, spinal injections of pain relieving drugs or operative intervention by spinal fusion, which all are lacking evidence for long-term improvement. Pharmacological pain relieving medicaments include opioids, non-opioids and non-steroidal anti-inflammatory drugs. The non-steroidal drugs used to relieve inflammatory pain mainly block the synthesis of PGE_2 with the disadvantage of also blocking other prostaglandins, which may have tissue protective function. Thus there is a significant need to develop new and more effective treatment options.

The use of natural herbs for medical treatments already has a long tradition. The great interest nowadays to use these herbs against various diseases is shown by the significant number of studies performed in the past decades. Over 5000 new studies are currently available investigating the beneficial effects of resveratrol in diseases such as inflammation, autoimmune diseases and cancer. In the current study, we could show evidence to support the positive properties of compounds of natural herbs namely resveratrol, curcuma (curcumin) and triptolide by demonstrating their preventative effects on inflammatory and catabolic events. For the *in vitro* experiments, we first pre-stimulated cultured IVD cells with IL-1β to provoke an inflammatory and catabolic cascade. With this method, all genes of interest (*IL-6*, *IL-8*, *MMP1*, *MMP3*, *MMP13* and *TLR2*) were significantly up-regulated on the mRNA level. The application of 50 μM of resveratrol, a phytoalexin found mostly in grapes, berries and peanuts, reduced gene expression with the highest reduction for *IL-6* (76%). We were able to confirm these results also on protein level for IL-6, IL-8, MMP1, MMP3 and MMP13, as determined by Western blot using precipitated proteins from the supernatant of IVD treated cells. We also demonstrated that resveratrol could decrease the active form of each measured MMP, indicating that resveratrol not only affects expression levels, but also depletes the activation of the pro-form of MMPs, thereby disturbing matrix breakdown. Investigating the underlying pathway, we focused on NF-κB and MAP kinases p38, ERK and JNK. We could not detect any intervention in these signalling pathways by resveratrol, as

determined using different methods such as immunofluorescence, EMSA and Western blot. Resveratrol not only showed anti-catabolic and anti-inflammatroy effects *in vitro*, but also demonstrated an analgesic potential in a rat model of painful radiculopathy. Comparisons of animal behavior were made between resveratrol treated and untreated groups in response to mechanical stimulation using von Frey filaments by evaluating hind paw withdrawal. The pain was evoked by application of autologous NP on the DRG of rats, which resulted in a lower threshold of the paw withdrawal after mechanical stimulus as compared to a sham control group. A lower threshold indicated that these rats were more sensitive to the mechanical stimulus. If co-application of NP tissue and resveratrol was performed at the DRG, rats were less sensitive to the mechanical stimulus as compared to the sham group, indicating a pain relieving effect of resveratrol *in vivo*. As production and release of pro-inflammatory cytokines seem to play a pivotal role in NP mediated pain and as resveratrol was able to reduce inflammatory and catabolic cell responses *in vitro* as well as pain *in vivo*, resveratrol may hold promise as a medical application for discogenic back pain.

Investigations conducted with curcuma, a yellow powder generated from dried rhizomes of *Curcuma longa*, also demonstrated interesting effects on IVD cells *in vitro*. IL-1β-induced increases in gene expression of *IL-6*, *MMP1*, *MMP3* and *MMP13* as well as *TLR2* were significantly decreased by application of 100 μg/ml curcuma, with an exception for *TNF-α*, which was even further up-regulated. The main components of curcuma were curcumin, demethoxycurcumin and bisdemethoxycurcumin, as confirmed by HPLC/MS measurements, with curcumin being present at the highest concentration (6 mg/ml of 320 mg/ml stock solution of curcuma). When treating IVD cells with curcumin (purchased from Sigma) at 20 μM, mRNA alterations were very comparable to effects observed upon curcuma treatment. Western blot as well as DNA binding assays revealed that the NF-κB pathway does not seem to play a role in the anti-inflammatory and anti-catabolic effect of curcumin observed in IVD cells. This was in contrast to a recent study in chondrocytes, where curcumin inhibited degradation of IκB and thus prevented p65 shuttling into the nucleus [266]. Curcumin instead showed an inhibitory effect on the MAP kinase JNK, although it had a stimulatory effect on p38 and ERK, as evaluated by Western blot analysis. The observed increase in *TNF-α* mRNA levels upon

curcuma/curcumin treatment could potentially be a consequence of the activated ERK or p38, but a causal relationship needs confirmation by further analyses. However, before considering curcumin for use in clinical trials, its analgesic effects need first to be demonstrated in animal models.

Triptolide also demonstrated potential beneficial effects for the treatment of disc-related pain. Triptolide is a diterpene lacton and an extract of the herb *Tripterygium wilfordii*. The application of 50 nM of triptolide to IVD cells *in vitro* exerted an inhibitory effect on IL-1β induced *IL-6*, *IL-8*, *MMP1*, *MMP2*, *MMP3* and *MMP13* gene expression. Furthermore, IL-1β pretreated IVD cells showed a reduced gene expression of structure proteins *Col-I* and *Col-II* and the proteoglycan *aggrecan*. Col-II and aggrecan synthesis was rescued and even enhanced by triptolide treatment. We could also detect reduced expression of *TLR2*, *TLR4* and *COX-2* by triptolide. Similar to curcuma/curcumin, we detected an up-regulation of *TNF-α* on the mRNA level. Furthermore, we also confirmed that triptolide had no influence on the NF-κB pathway, as demonstrated by immunocytostaining and Western blot analysis. However, it did induce a strong inhibitory effect on p38 activation, and a slight effect on ERK pathway transduction.

As bioavailability and diffusion rates are not known *in vivo*, our results obtained in this *in vitro* study only provide first evidence for a possible application in disc disease. Particularly, the observed *TNF-α* increase needs to be examined and further investigated.

All together, resveratrol, curcuma as well as triptolide showed anti-inflammatory and anti-catabolic effects in IVD cells *in vitro*. With resveratrol, we were additionally able to demonstrate an analgesic effect *in vivo.*

Clinical trials for a variety of applications (but not IVD disease) are being performed for all three tested substances. 63 studies are currently running for resveratrol, 75 for curcumin and 1 for triptolide, conducted in different countries (http://clinicaltrials.gov). Resveratrol is tested e.g. for cardiovascular diseases, memory, friedreich ataxia, diabetes type II, obesity, colon cancer and Alzheimer's disease. Curcumin is tested for colon cancer, dermatitis, multiple myeloma, irritable bowel syndrome, Alzheimer's disease, multiple sclerosis, depression, osteosarcoma, RA and OA. One clinical trial for triptolide is currently conducted in East Asia for the treatment of polycystic kidney diseases at the status of recruiting participants.

Current treatments of discogenic back pain are either conservative or operative. So far, both methods do not provide the anticipated improvement in the long term. For instance, pain relieving pharmacological treatments do not address the problem of IVD degeneration. Surgical interventions, such as spinal fusion, have the downside of a loss of flexibility and movement. Furthermore, adjacent discs are more prone to degeneration due to altered biomechanics and increased strain [267-269]. To overcome these problems, alternative methods of treatment are being investigated either *in vitro*, by using explants IVD culture models, or by conducting *in vivo* studies. Biological treatment options currently under investigation include either injections of growth factors or stem cell therapy. So far, results were only positive for small animals, but are not yet distinct for large animals [270-272].

The investigated natural herbs in this work may provide another treatment strategy. Compounds such as resveratrol, curcuma or triptolide promise an alternative treatment option for discogenic back pain, as demonstrated by their anti-inflammatory, anti-catabolic and anabolic effect *in vitro*, as well as analgesic potential *in vivo*. Thus, a better understanding of the source and the underlying pathway involved in the progression of a painful degenerated disc could help in development of more precise treatment strategies.

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